

# THE PATHOGENIC ROLE OF EOSINOPHILS IN AUTOIMMUNE MYOCARDITIS AND INFLAMMATORY DILATED CARDIOMYOPATHY

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## Abstract

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Myocarditis is a rare but potentially devastating inflammatory disease of the heart. Severity can vary widely from asymptomatic to fulminant disease and sudden death. Up to 30% of myocarditis patients go on to develop inflammatory dilated cardiomyopathy (DCMi), which is a major cause of heart failure in children and young adults. Patients with eosinophilia frequently develop cardiomyopathy, suggesting a pathogenic role for eosinophils in the heart. We used the experimental autoimmune myocarditis (EAM) model in different genetically modified mice to determine the role of eosinophils in myocarditis and DCMi. Using adoptive transfer experiments and  $CCR3^{-/-}$  mice, we found that the eotaxin-CCR3 pathway was required for eosinophil trafficking to the heart in mice with eosinophilic myocarditis. We identified cardiac fibroblasts as the source of eotaxin-1 and infiltrating macrophages as the source of eotaxin-2. Eotaxins were also increased in patients with eosinophilic myocarditis compared to chronic lymphocytic myocarditis and eotaxin expression was positively correlated with the number of heart-infiltrating eosinophils. We then showed that eosinophils contribute to myocarditis pathology. Eosinophils were dispensable for myocarditis induction but required for progression to DCMi. Eosinophil-deficient  $\Delta dbpGATA1$  mice, in contrast to WT mice, showed no signs of heart failure by echocardiography. Induction of EAM in hypereosinophilic IL-5Tg mice resulted in eosinophilic myocarditis with severe atrial inflammation, which progressed to severe DCMi. This was not a direct effect of IL-5 as IL-5Tg $\Delta dbpGATA1$  mice were protected from DCMi while IL-5 $^{-/-}$  mice exhibited DCMi comparable to WT mice. Eosinophils drove progression to DCMi through their production of IL-4. Our experiments showed eosinophils were the major IL-4 expressing cell type in the heart during EAM, IL-4 $^{-/-}$  mice were protected from DCMi like  $\Delta dbpGATA1$  mice, and eosinophil-specific IL-4 deletion resulted in improved heart function. In conclusion, eosinophils drive progression of myocarditis to DCMi, cause severe DCMi when present in large numbers, and mediate this process through IL-4.

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## Preface

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## Table of Contents

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|   |      |
|---|------|
| Abstract.....   | ii   |
| Preface .....   | iv   |
| List of Tables.....   | xiii |
| List of Figures .....   | xiv  |
| Chapter 1: Introduction .....   | 1    |
| Myocarditis.....  | 1    |
| Eosinophilic myocarditis.....   | 4    |
| Dilated cardiomyopathy .....  | 7    |
| Experimental autoimmune myocarditis .....   | 10   |
| Eosinophils.....  | 11   |
| Chapter 2: Epidemiology of myocarditis and dilated cardiomyopathy .....             | 14   |
| Myocarditis.....  | 14   |
| Global burden of myocarditis.....   | 14   |
| Natural history of myocarditis.....   | 16   |
| Fatal myocarditis .....   | 18   |
| Sex and age differences .....   | 20   |
| Chagas.....   | 21   |
| Myocarditis as a rare complication in common infectious diseases and vaccines ..... | 22   |
| Lymphocytic myocarditis .....   | 23   |

|  |    |
|--|----|
| Eosinophilic myocarditis.....  | 24 |
| Mortality in eosinophilic myocarditis .....                                      | 25 |
| Hypersensitivity myocarditis .....   | 27 |
| Hypersensitivity myocarditis in heart transplantations .....                     | 27 |
| Tropical endomyocardial fibrosis .....   | 28 |
| Eosinophilic granulomatosis with polyangiitis.....                               | 29 |
| Hypereosinophilic syndrome.....  | 29 |
| Giant cell myocarditis .....   | 30 |
| Dilated cardiomyopathy .....   | 30 |
| Dilated cardiomyopathy in children .....   | 31 |
| Conclusions.....   | 32 |
| Chapter 3: Eosinophil trafficking to the heart in eosinophilic myocarditis ..... | 33 |
| Summary .....  | 33 |
| Introduction.....  | 34 |
| Methods .....  | 35 |
| Patients.....  | 35 |
| Animals .....  | 36 |
| Experimental autoimmune myocarditis.....   | 36 |
| Eosinophil isolation.....  | 36 |
| Flow cytometry and cell sorting .....  | 37 |

|   |    |
|---|----|
| Cardiac fibroblast isolation and culture .....  | 38 |
| Bone marrow-derived macrophage culture .....  | 38 |
| Quantitative Real-time PCR.....   | 38 |
| ELISA .....   | 39 |
| Histology and immunohistochemistry .....  | 39 |
| Statistics.....   | 39 |
| Results .....   | 40 |
| Eosinophils localize to the heart in response to eotaxin-CCR3 signaling .....   | 40 |
| Eosinophils reach highest frequency in the hearts of IFN $\gamma$ <sup>-/-</sup> IL-17A <sup>-/-</sup> $\Delta$ dbpGATA1 mice.....  | 41 |
| Genetic ablation of CCR3 in IFN $\gamma$ <sup>-/-</sup> IL-17A <sup>-/-</sup> mice blocks eosinophil trafficking to the heart ..... | 45 |
| CCL11 and CCL24 are expressed by different cell types in the heart.....   | 45 |
| Cardiac fibroblasts are major CCL11 producers in the heart.....   | 47 |
| CCL24 is produced by F4/80 <sup>+</sup> macrophages during myocarditis .....  | 47 |
| Primary cardiac fibroblasts produce eotaxins in vitro in response to IL-4 and IL-13 .....   | 53 |
| IL-4 and IL-13 induce CCL24 in macrophages .....  | 54 |
| Cardiac eotaxin expression is increased in patients with eosinophilic myocarditis.....  | 55 |
| Discussion .....  | 57 |
| Chapter 4: Eosinophil-derived IL-4 drives heart failure.....  | 61 |
| Summary .....   | 61 |
| Introduction.....   | 62 |

|  |    |
|--|----|
| Materials and Methods .....  | 63 |
| Patients.....  | 63 |
| Mice.....  | 64 |
| Induction of EAM.....  | 64 |
| Assessment of EAM severity and fibrosis.....   | 64 |
| Light microscopy.....  | 65 |
| Electron microscopy .....  | 65 |
| Echocardiography.....  | 65 |
| Flow cytometry.....  | 66 |
| Isolation of primary adult mouse cardiomyocytes .....  | 66 |
| Hydroxyproline assay.....  | 66 |
| ELISA .....  | 67 |
| Quantitative PCR .....   | 67 |
| Statistics.....  | 67 |
| Results .....  | 68 |
| Eosinophil-deficient mice develop myocarditis but are protected from dilated cardiomyopathy..... | 68 |
| $\Delta$ dblGATA1 mice develop fibrosis but show altered tissue remodeling .....                 | 69 |
| Lack of eosinophils does not alter cardiac inflammatory infiltrate in EAM.....                   | 73 |
| IL-5Tg mice develop eosinophilic myocarditis.....  | 75 |
| Eosinophilic atrial inflammation progresses to severe fibrosis .....                             | 79 |

|   |     |
|---|-----|
| Eosinophils drive severe DCMi .....   | 79  |
| IL-5 is dispensable for progression to DCMi .....   | 81  |
| Eosinophils do not cause DCMi through effects on dendritic cells, antibody production, or cytotoxic effects ..... | 83  |
| Eosinophils in the heart are activated .....  | 85  |
| Eosinophils account for the majority of IL-4 expressing cells in EAM .....  | 88  |
| IL-4 <sup>-/-</sup> mice are protected from DCMi.....   | 89  |
| Eosinophil-specific deletion of IL-4 ameliorates DCMi.....  | 89  |
| Discussion .....  | 93  |
| Chapter 5: Eosinophils in autoimmune diseases .....   | 98  |
| Summary .....   | 98  |
| Introduction.....   | 98  |
| Possible eosinophil effector functions in autoimmune diseases .....   | 99  |
| Role of eosinophils in autoimmune diseases.....   | 102 |
| Bullous pemphigoid .....  | 102 |
| Inflammatory Bowel diseases .....   | 103 |
| Eosinophilic granulomatosis with polyangiitis.....  | 106 |
| Eosinophilic myocarditis .....  | 108 |
| Neuromyelitis optica.....   | 110 |
| Primary biliary cirrhosis .....   | 112 |
| Other diseases.....   | 113 |

|   |     |
|---|-----|
| Autoimmune diseases in patients with eosinophil-associated diseases ..... | 115 |
| Hypereosinophilic syndrome.....   | 116 |
| Eosinophilic gastrointestinal diseases.....                               | 116 |
| Asthma.....   | 117 |
| Conclusions.....  | 117 |
| Future research needs .....   | 119 |
| Chapter 6: Conclusions and future directions .....                        | 121 |
| References .....  | 126 |
| Curriculum vitae .....  | 162 |



## List of Tables

---

|  |     |
|--|-----|
| Table 1: Subtypes of myocarditis.....                                    | 3   |
| Table 2: Subtypes of eosinophilic myocarditis .....                      | 4   |
| Table 3: Possible etiologies of eosinophilic myocarditis .....           | 5   |
| Table 4: Etiologies of dilated cardiomyopathy .....                      | 9   |
| Table 5: Reported mortality in eosinophilic myocarditis .....            | 26  |
| Table 6: Patient characteristics .....                                   | 35  |
| Table 7: Primer sequences .....  | 68  |
| Table 8: Autoimmune diseases with potential eosinophil involvement ..... | 114 |

## List of Figures

---

|   |    |
|---|----|
| Figure 1: Autoimmune myocarditis can progress to inflammatory dilated cardiomyopathy.....   | 8  |
| Figure 2: Model of experimental autoimmune myocarditis in BALB/c mice.....  | 11 |
| Figure 3: Cellular structure, receptors and mediators of eosinophils.....   | 13 |
| Figure 4: Estimated disability-adjusted life years lost due to cardiomyopathy and myocarditis in 2015 ....  | 15 |
| Figure 5: Possible outcomes of myocarditis.....   | 16 |
| Figure 6: CCR3 is required for eosinophil trafficking to the heart in eosinophilic myocarditis.....   | 42 |
| Figure 7: Gating strategy of eosinophils.....   | 43 |
| Figure 8: Adoptively transferred eosinophils reach highest frequency in the heart.....  | 44 |
| Figure 9: Genetic ablation of CCR3 in IFN $\gamma$ <sup>-/-</sup> IL-17A <sup>-/-</sup> mice reduces eosinophil frequency in the heart.....                                       | 46 |
| Figure 10: CCL11 is expressed by cardiac fibroblasts while CCL24 is expressed by F4/80 <sup>+</sup> macrophages during eosinophilic myocarditis .....                             | 48 |
| Figure 11: Gating strategy for prime flow RNA assay .....   | 50 |
| Figure 12: Flow sorting of cardiac resident and cardiac infiltrating cells .....  | 51 |
| Figure 13: Gating strategy for flow sorting of heart infiltrating and resident cell populations.....  | 52 |
| Figure 14: Ccl11 expression in sorted cells.....  | 53 |
| Figure 15: IL-4 and IL-13 induce CCL11 in cardiac fibroblasts and CCL24 in macrophages .....  | 54 |
| Figure 16: Eotaxins are increased in the hearts of patients with eosinophilic myocarditis .....   | 56 |
| Figure 17: Model of eosinophil trafficking to the heart during eosinophilic myocarditis .....   | 57 |
| Figure 18: Eosinophil-deficient mice develop EAM, but are protected from DCMi.....  | 70 |
| Figure 19: Protection of $\Delta$ dbpGATA1 mice from DCMi is accompanied by changes in tissue remodeling-associated genes not changes in heart-infiltrating cell populations..... | 71 |
| Figure 20: Lack of eosinophils does not alter inflammatory infiltrate in EAM .....  | 74 |
| Figure 21: IL-5 transgenic mice develop severe eosinophilic myocarditis.....  | 76 |

|   |     |
|---|-----|
| Figure 22: Atrial versus ventricular inflammation in IL-5Tg mice and an eosinophilic myocarditis patient .  | 77  |
| Figure 23: IL-5Tg but not IL-5Tg $\Delta$ dblGATA1 or IL-5 <sup>-/-</sup> mice progress to severe DCMi.....   | 80  |
| Figure 24: Eosinophils rather than IL-5 contribute to severe DCMi and atrial inflammation / fibrosis .....  | 81  |
| Figure 25: Eosinophils do not drive DCMi through effects on dendritic cells, antibody production, cytotoxic effects on cardiomyocytes or eosinophil peroxidase.....                       | 84  |
| Figure 26: Heart-infiltrating eosinophils show an activated phenotype. ....   | 86  |
| Figure 27: Electron microscopy of heart-infiltrating and blood eosinophils .....  | 88  |
| Figure 28: Eosinophils are the major producers of IL-4 in myocarditis.....  | 90  |
| Figure 29: Eosinophil-derived IL-4 promotes DCMi .....  | 91  |
| Figure 30: EoCre <sup>wt/tg</sup> IL-4/13 <sup>fl/fl</sup> mice develop fibrosis to the same extent as WT mice but show altered tissue remodeling similar to $\Delta$ dblGATA1 mice ..... | 92  |
| Figure 31: Possible eosinophil effector mechanisms in autoimmune diseases.....  | 101 |
| Figure 32: Possible mechanisms of eosinophil-mediated damage in autoimmune diseases.....  | 120 |

## Chapter 1: Introduction

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### Myocarditis

An older, widely-used definition of myocarditis are the so-called *Dallas criteria*<sup>1</sup>. This histopathological categorization requires an inflammatory infiltrate of the heart muscle with associated myocyte damage or necrosis in the absence of an ischemic event. In 1995, myocarditis was defined as “*an inflammatory disease of the myocardium, diagnosed by established histological, immunological, and immunohistochemical criteria*” by the World Health Organization / International Society and Federation of Cardiology (ISFC). Inflammatory cardiomyopathy is myocarditis with an associated cardiac dysfunction. These definitions have more recently been reiterated and refined by the European Society of Cardiology (ESC)<sup>2</sup>. Histological criteria were defined as “*histological evidence of inflammatory infiltrates within the myocardium associated with myocyte degeneration and necrosis of non-ischaemic origin*” in accordance with the Dallas criteria<sup>1</sup>. The immunohistochemical criteria for an abnormal inflammatory infiltrate were defined as “*≥ 14 leucocytes/mm<sup>2</sup> including up to 4 monocytes/mm<sup>2</sup> with the presence of CD3 positive T-lymphocytes ≥ 7 cells/mm<sup>2</sup>*”.

These definitions require an endomyocardial biopsy (or postmortem autopsy) and histological analysis of tissues to determine the presence of infiltrating immune cells. Moreover, special subtypes of myocarditis with unique prognoses and treatment can often not be diagnosed by noninvasive testing<sup>3</sup>. The complication rate is low and, most importantly, the infection status, which determines treatment strategy, can only be determined with an endomyocardial biopsy<sup>2</sup>. However, endomyocardial biopsies are not universally employed for the diagnosis of myocarditis. Critics argue that the sensitivity is low and that complications and costs are not justified<sup>4</sup>. A common statement by the American Heart Association, American College of Cardiology and the European Society of Cardiology delineates specific clinical circumstances that justify endomyocardial biopsies<sup>3</sup>. These include new-onset heart failure with hemodynamic compromise, relative

recent heart failure with additional complication or nonresponse to standard treatments, unexplained cardiomyopathy in children, and several other conditions. Noninvasive diagnostic approaches include the use of echocardiography, electrocardiograms, cardiac magnetic resonance imaging, and rarely positron emission tomography scans<sup>2</sup>. Clinical symptoms of myocarditis include acute chest pain, new-onset or worsening of dyspnea, fatigue, signs of heart failure, palpitations, unexplained arrhythmias, aborted sudden cardiac death or unexplained cardiogenic shock. Patients may also be asymptomatic in the presence of myocardial inflammation<sup>5,6</sup>.

Treatment strategies depend on the clinical symptoms of the patient, the suspected etiology and the disease state<sup>2</sup>. Conventional medical treatment of heart failure or arrhythmias is generally undertaken. Unstable patients will be treated in intensive care units with respiratory and mechanical support, and fulminant cases may require ventricular assist devices or extracorporeal membrane oxygenation to bridge the patient to recovery or heart transplantation<sup>7, 8</sup>. Immunosuppressive therapy is indicated in virus-negative autoantibody-positive patients, where it was shown to benefit a majority of patients<sup>9</sup>. Immunosuppressive drugs are also used in patients with specific etiologies such as giant cell myocarditis or eosinophilic myocarditis.

The main forms of pathogenesis in myocarditis are thought to be viral infection and autoimmunity. These forms are not mutually exclusive, as viral infection may progress to post-infectious autoimmune disease and patients may have both, viral genomes in endomyocardial biopsies and anti-cardiac autoantibodies<sup>10, 11</sup>. Autoantibodies against cardiac antigens are present in the majority of myocarditis patients<sup>10</sup>. Moreover, myocarditis is associated with other autoimmune diseases<sup>12</sup>.

Myocarditis can be classified based on histology, etiology, disease stage or extent of myocyte damage (Table 1). These classifications are partially overlapping and different studies may report different sets of classifications, making it often difficult to compare outcomes. The histological subtypes are based on the predominant infiltrating cell type detected on endomyocardial biopsies or at autopsy. Lymphocytes

accompanied by numerous other cell types predominate in lymphocytic myocarditis. The classification of eosinophilic myocarditis seems to be variable with “any”, “numerous” or “predominantly” eosinophils being sufficient for the diagnosis. Giant cell myocarditis has very characteristic, large, multinucleated cells derived from monocytes and usually severe myocyte necrosis. The etiologies show partial overlap with the histological appearance. For example, viral myocarditis is usually thought to be predominantly lymphocytic while hypersensitivity myocarditis and parasitic myocarditis are generally eosinophilic and giant cell myocarditis is thought to be an autoimmune disease<sup>13, 14</sup>. However, lymphocytic myocarditis can also be autoimmune-mediated. In addition, there is often no identified etiology – many cases of myocarditis are idiopathic<sup>5, 6</sup>.

**Table 1: Subtypes of myocarditis**

| Classification          | Subtypes  |
|-------------------------|---|
| Based on histology      | Lymphocytic myocarditis<br>Neutrophilic/ granulomatous<br>Eosinophilic myocarditis<br>Giant cell myocarditis<br>Sarcoidosis                     |
| Based on etiology       | Infectious<br>- viral (see <sup>13</sup> )<br>- bacterial<br>- parasitic (see Table 3)<br>Autoimmune<br>Toxic<br>Hypersensitivity<br>Idiopathic |
| Based on disease stage  | Acute myocarditis<br>Chronic myocarditis<br>Healed myocarditis  |
| Based on myocyte damage | Myocarditis (inflammation and myocyte damage/necrosis)<br>Borderline myocarditis (mild inflammation only)                                       |

This table is based on<sup>1, 2</sup>.

## Eosinophilic myocarditis

Eosinophilic myocarditis is a relatively rare subtype of myocarditis that is characterized by eosinophil infiltration in the myocardium. The diagnosis is made in a similar way as the diagnosis for myocarditis, with the additional criterion of eosinophilic inflammation in the myocardium by histological examination. Often, there is an associated increase in blood eosinophils, which may raise the suspicion of eosinophilic myocarditis<sup>14</sup>. However, eosinophilia is not always present<sup>15, 16</sup>. Eosinophilic myocarditis is usually treated with immunosuppressive therapy, or based on the underlying etiology<sup>17</sup>. Eosinophilic myocarditis can be classified according to histopathological characteristics, suspected etiology, or associated clinical conditions (Table 2, Table 3). These classifications are partially overlapping and do not encompass all cases, revealing the incomplete appreciation of the pathogenesis of eosinophilic myocarditis.

**Table 2: Subtypes of eosinophilic myocarditis**

| Type   | Description  |
|--|--|
| Hypersensitivity myocarditis   | Allergic reaction (delayed hypersensitivity reaction) to drugs (see Table 3), rather than toxic effects of drugs; often typical signs of allergic reactions (fever, rash, blood eosinophilia); can result in sudden death; patchy myocarditis with interstitial and perivascular eosinophil infiltration |
| Acute necrotizing eosinophilic myocarditis                           | Severe, acute form that may be rapidly fatal; may represent the extreme form of a hypersensitivity myocarditis, but generally the etiology is unknown; intense, diffuse eosinophil and lymphocyte infiltration with marked myocardial necrosis   |
| Eosinophilic myocarditis associated with hypereosinophilic syndromes | Eosinophilic endomyocarditis in patients with prolonged high levels of blood eosinophils such as in hypereosinophilic syndrome or eosinophilic granulomatosis with polyangiitis; characterized by endomyocardial fibrosis, mural thrombus formation and valvular dysfunction                             |
| Tropical endomyocardial fibrosis                                     | Unknown etiology, hypotheses include helminth-induced chronic eosinophilia leading to endomyocardial fibrosis similar to the process seen in hypereosinophilic syndromes, or toxic agents  |

This table is based on<sup>14, 18, 19</sup>.

Hypersensitivity myocarditis is thought to be an allergic delayed type hypersensitivity reaction to drugs, rather than a toxic effect<sup>14</sup>. Some patients present with typical signs of an allergic reaction, such as fever, rash, and blood eosinophilia<sup>20, 21</sup>. Given these unspecific symptoms, hypersensitivity myocarditis may not be recognized and can result in sudden death. The first description came from French and Weller, who described eosinophilic myocarditis in patients taking sulfonamides within weeks before death<sup>22</sup>. They also showed that sulfonamides administered to rats or mice resulted in a similar eosinophilic myocarditis. Numerous drugs have since been associated with eosinophilic myocarditis (Table 3)<sup>14, 18, 20, 23</sup>. When patients who have recently started taking one of the implicated drugs develops symptoms of an allergic reaction, chest pain, or symptoms of heart failure, an endomyocardial biopsy can confirm the presence of hypersensitivity myocarditis<sup>14, 24</sup>. The drug causing the allergic reaction should then immediately be stopped and heart failure is treated with standard therapy. Corticosteroids are often initiated as well<sup>25</sup>.

**Table 3: Possible etiologies of eosinophilic myocarditis**

| Group        | Etiological agents   |
|--------------|--|
| Drugs        | Acetazolamide, amitriptyline, amphotericin B, ampicillin, carbamazepine, cefaclor, chloramphenicol, chlorthalidone, clozapine, desipramine, hydrochlorothiazide, indomethacin, interleukin (IL)-4, isoniazid, methyldopa, oxyphenylbutazone, para-aminosalicylic acid, penicillin, phenindione, phenobarbital, phenylbutazone, phenytoin, spironolactone, streptomycin, sulfadiazine, sulfisoxazole, sulfononylureas, tetanus toxoid, tetracycline |
| Parasites    | <i>Trypanosoma cruzi</i> , <i>Toxoplasma gondii</i> , <i>Trichinella spiralis</i> , <i>Entamoeba fragilis</i> , <i>Isospora belli</i> , <i>Strongyloides spp.</i> , <i>Toxocara canis</i> , <i>Toxocara cati</i> , Baylisascaris procyonis, <i>Echinococcus</i> , and <i>Schistosoma spp.</i> , visceral larva migrans of certain Nematode species   |
| Eosinophilia | Hypereosinophilic syndrome, eosinophilic granulomatosis with polyangiitis  |

This table is based on<sup>14, 19, 23, 24, 26</sup>.

Acute necrotizing eosinophilic myocarditis is a very severe form of eosinophilic myocarditis. It presents with an acute onset of fever, rash, severe chest pain, and heart failure<sup>27, 28</sup> and may simply represent the most



severe forms of hypersensitivity myocarditis<sup>28</sup>. Acute necrotizing eosinophilic myocarditis is also found in patients with drug rash with eosinophilia and systemic symptoms (DRESS)<sup>29, 30</sup>, a general term for severe allergic reactions to drugs with systemic involvement that may also affect other organs than the heart. Given the severe heart failure symptoms and high mortality, patients are treated aggressively with high doses of corticosteroids and sometimes additional mechanical support such as ventricular assist devices or extracorporeal membrane oxygenation. If patients survive the acute phase, complete recovery is possible<sup>29, 31</sup>.

Eosinophilic myocarditis occurs frequently in patients with prolonged hypereosinophilia (blood eosinophils >1500/ $\mu$ l), as in hypereosinophilic syndrome (HES) and eosinophilic granulomatosis with polyangiitis (EGPA). Cardiac involvement in these patients is a major contributing factor to morbidity and mortality<sup>32, 33</sup>. HES usually causes eosinophilic endomyocardial fibrosis and thrombus formation<sup>33, 34</sup>. The disease is thought to evolve in 3 stages, beginning with acute myocarditis with myocyte necrosis, vascular inflammation, and damage of the endocard by eosinophils. In the second stage thrombi form on the damaged endocard and can obstruct large parts of the ventricles. Finally, thrombi are replaced by fibrosis which may also involve the valves<sup>33</sup>. Patients often don't show symptoms in the first stage and may not develop heart failure until the final stage, at which point both restrictive and dilated cardiomyopathies are possible<sup>33</sup>. Patients with EGPA can develop eosinophilic myocarditis, eosinophilic vasculitis of the heart and endomyocardial fibrosis<sup>35</sup>.

Eosinophilic myocarditis in association with parasitic infections is thought to be a result of sustained high blood eosinophilia rather than direct infection of the heart with parasites<sup>36</sup>, although some parasites can directly infect the heart<sup>26, 37</sup>. Parasites that may cause eosinophilic myocarditis are listed in Table 3. Tropical endomyocardial fibrosis accounts for a striking proportion of heart failure in some endemic regions of Sub-Saharan Africa and was also reported in South America and Asia<sup>38</sup>. The clinical presentations are similar to

those of patients with HES<sup>19,38</sup>. Etiology is unknown and hypotheses range from infectious (parasitic) causes over nutritional deficiencies and toxins to the damaging effect of prolonged eosinophilia<sup>38</sup>.

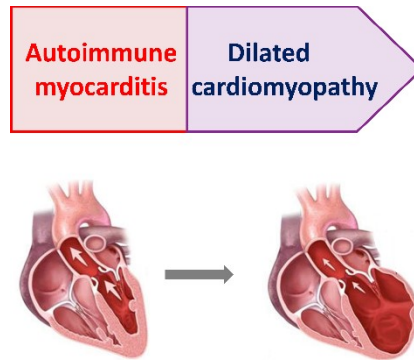
The high frequency of endomyocardial damage in patients with prolonged eosinophilia and cases of acute necrotizing myocarditis where eosinophils are found next to necrotic myocytes have given rise to the idea that eosinophils may be particularly pathogenic in the heart<sup>39-42</sup>. Several mechanisms have been proposed. Eosinophils may be directly cytotoxic to cardiomyocytes<sup>39,43,44</sup>, activate cardiac mast cells<sup>45</sup>, or release pro-thrombotic tissue factor<sup>46</sup>. However, these studies are not mechanistic and the true pathological effects of eosinophils in the heart remain to be discovered.

### **Dilated cardiomyopathy**

Dilated cardiomyopathy (DCM) is a clinical diagnosis characterized by *“dilation and impaired contraction of the left or both ventricles that is not explained by abnormal loading conditions or coronary artery disease. DCM includes idiopathic, familial/genetic, viral and/or immune, alcoholic/toxic subtypes.”*<sup>2</sup>. This recent definition from the World Health Organization / International Society and Federation of Cardiology highlights the large and heterogenous group of disorders defined by a common morphological abnormality and the failure of myocardial performance. Diagnosis is made by echocardiography or cardiac magnetic resonance imaging showing a decrease in left ventricular ejection fraction and dilation of the left ventricle at the end of diastole to a Z score of >2 standard deviations<sup>47</sup>.

One of the causes of DCM is myocarditis, which is responsible for about 9-16% of cases with new onset DCM (see Figure 1 and discussion in Chapter 2)<sup>48-50</sup>. How myocarditis progresses to DCM is not completely understood. Replacement of the inflammatory infiltrate with fibrotic tissue, destruction of myocytes, and remodeling of the extracellular matrix likely play a role<sup>51</sup>. There are also numerous other etiologies of DCM: genetic diseases, drugs, toxins, endocrinological diseases, nutritional deficiencies, electrolyte disturbance, infections, autoimmune diseases (including myocarditis), and peripartum cardiomyopathy (Table 4).

Monogenetic traits as well as complex genetic traits are the most common cause of DCM<sup>52</sup>. Familial disease accounts for about 20-35% of cases<sup>53</sup>. Genetic predisposition can also interact with environmental factors<sup>47</sup>.



**Figure 1: Autoimmune myocarditis can progress to inflammatory dilated cardiomyopathy**

Clinical evaluation of patients with suspected DCM includes a detailed medical history, family history for the evaluation of genetic causes, and detailed physical examination which may provide clues about the etiology as well as the severity of disease and the patient's prognosis<sup>54</sup>. Diagnostic tests include complete blood counts, urinalysis, serum electrolytes, liver function tests, biomarkers, etc. Imaging and assessment of cardiac function are also done through electrocardiograms, echocardiograms, and sometimes magnetic resonance imaging<sup>47, 54</sup>. The latter allows visualization of inflammatory processes or scars in the myocardium which will be the case in DCM caused by myocarditis. In patients with clinically suspected myocarditis, endomyocardial biopsies are recommended<sup>47</sup>.

**Table 4: Etiologies of dilated cardiomyopathy**

| <b>Group</b>              | <b>Agent / subtype / disease</b>  |
|---------------------------|---|
| Genetic                   | Titin, lamin A/C, myosin heavy chain, troponin T, myosin-binding protein C, RNA-binding motif-20, myopalladin, sodium voltage-gated channel alpha subunit 5, BaCl2-associated athanogene 3, phospholamban, dystrophin, tafazin  |
| Drugs                     | Anthracyclines, antimetabolites, alkylating agents, Taxol, hypomethylating agents, monoclonal antibodies, tyrosine kinase inhibitors, immunomodulating agents, clozapine, olanzapine, chlorpromazine, risperidone, lithium, methylphenidate, tricyclic antidepressants, Chloroquine, all-trans retinoic acid, antiretroviral agents, phenothiazines |
| Toxins                    | Ethanol, cocaine, amphetamines, ecstasy, arsenic, cobalt, steroids  |
| Nutritional deficiencies  | Selenium deficiency, thiamine deficiency, zinc and copper deficiency, carnitine deficiency, iron overload   |
| Electrolyte disturbance   | Hypocalcemia, hypophosphatemia  |
| Endocrinological diseases | Hypothyroidism, hyperthyroidism, Addison disease, pheochromocytoma, acromegaly, diabetes mellitus   |
| Infection                 | Viral, bacterial, mycobacterial, fungal, parasitic  |
| Autoimmune diseases       | Autoimmune myocarditis, polymyositis, dermatomyositis, eosinophilic granulomatosis with polyangiitis, granulomatosis with polyangiitis, systemic lupus erythematosus, sarcoidosis   |
| Peripartum                | Peripartum dilated cardiomyopathy   |

This table was adapted from<sup>47</sup>.

Therapy follows the general recommendation for heart failure patients by the American College of Cardiology and the American Heart Association<sup>54</sup> or the European Society of Cardiology guidelines for chronic heart failure<sup>55</sup>. Specific etiologies may warrant additional treatments. For example, genetic counseling and screening of relatives is conducted in genetic forms of DCM. DCM caused by myocarditis should be treated according to current guidelines for systolic heart failure and additional

immunosuppression in the case of eosinophilic or giant cell myocarditis and cardiac sarcoidosis<sup>56</sup>. Intracardiac devices may be implanted in DCM patients to prevent sudden cardiac death<sup>54</sup>.

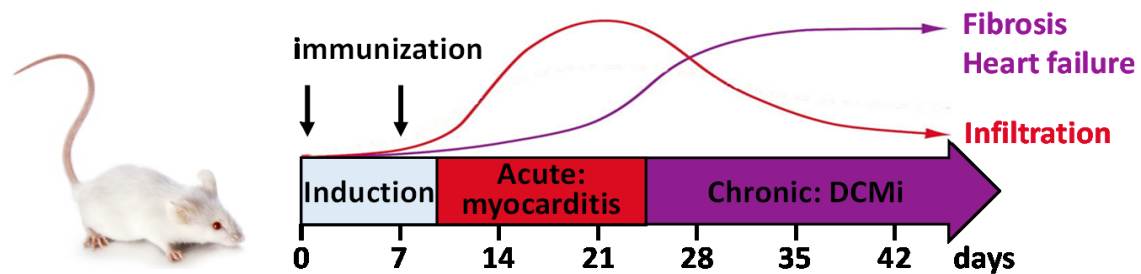
### **Experimental autoimmune myocarditis**

Animal models are necessary to study the complex interplay between the immune system and the heart to generate mechanistic insights that will eventually improve diagnosis and therapy of myocarditis in humans. The main mouse models of myocarditis are Coxsackievirus B3 (CVB3) myocarditis and experimental autoimmune myocarditis (EAM).

The murine CVB3 myocarditis model has been used to study viral myocarditis for over 40 years<sup>57</sup>. Depending on the host strain, CVB3 can induce transient or persistent myocarditis of different severity<sup>58</sup>. Strains with persistent myocarditis also develop autoantibodies against cardiac antigens. This led to the development of the EAM model, where mice are immunized with cardiac myosin in Complete Freund's Adjuvant (CFA) generating an autoimmune response against the heart resulting in autoimmune myocarditis<sup>59</sup>.

Since its development in the 1980s the EAM model has been refined and today synthetic immunodominant peptides are used for the induction of autoimmune myocarditis<sup>60</sup>. The immunodominant peptide for BALB/c mice is the cardiac myosin heavy chain  $\alpha$  peptide 614-629 (SLKLMATLFSTYASAD)<sup>61</sup>. To induce disease, 100  $\mu$ g peptide is emulsified in CFA supplemented to 5mg/ml with heat-killed *Mycobacterium tuberculosis* and injected subcutaneously on days 0 and 7 into susceptible mouse strains. On day 0, mice also receive an intraperitoneal injection of 500 ng pertussis toxin<sup>60</sup>. The use of CFA on both day 0 and day 7 is absolutely required to induce disease (Jillian Fontes, Jobert Barin, Monica Talor, Noel Rose and Daniela Čiháková, unpublished observations). Substituting CFA for either incomplete Freund's Adjuvant or Alum adjuvants in either or both injections is not sufficient to induce disease, highlighting the strong inherent tolerance to cardiac antigens that has to be overcome.

In our studies, BALB/c mice are used, which develop myocarditis of intermediate severity and progress to a chronic stage with DCM (Figure 2)<sup>12</sup>. The first 10 days are the induction phase during which an adaptive immune response to cardiac myosin is established. Infiltration of the myocardium with immune cells begins around day 10-12, marking the beginning of the acute myocarditis phase. Autoreactive T cells as well as numerous other immune cells infiltrate the heart and autoantibodies can be detected in the serum. Inflammation peaks around day 21 post immunization and declines thereafter. In most mice inflammation resolves thereafter, but occasionally chronic inflammation can be observed as late as day 60. Following the acute myocarditis phase, inflammation is replaced by fibrosis and activation of tissue remodeling processes. During the chronic phase mice develop DCM, which can be detected by echocardiography.



**Figure 2: Model of experimental autoimmune myocarditis in BALB/c mice**

At days 0 and 7, mice are immunized with cardiac myosin heavy chain  $\alpha$  peptide 614-629 in Complete Freund's Adjuvant. On day 0, mice also receive 500 ng pertussis toxin. Myocarditis and DCM develop in 3 phases: induction, acute myocarditis, and chronic DCM. Abbreviations: DCMi, inflammatory dilated cardiomyopathy.

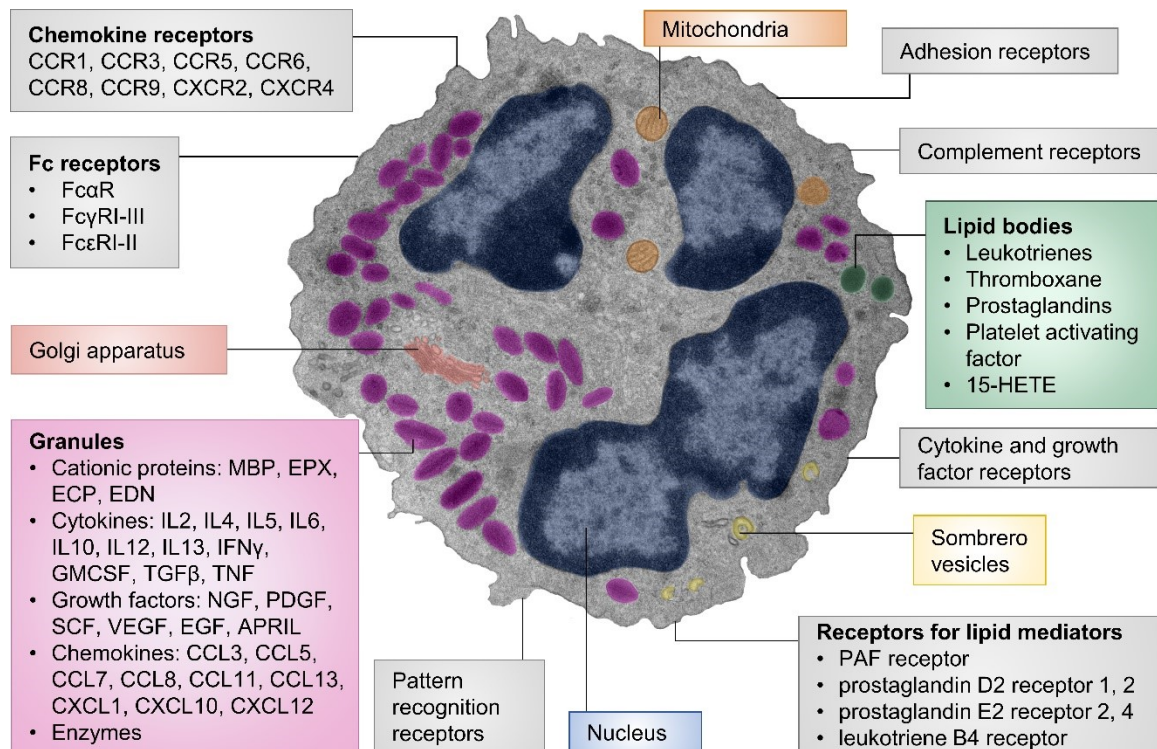
## Eosinophils

Eosinophils are multifunctional granulocytes that develop in the bone marrow in response to IL-5, with a minor role for IL-3, GM-CSF, and IL-33<sup>62-65</sup>. IL-5 also mediates the release of mature eosinophils into the bloodstream from where they migrate into tissues<sup>66</sup>. In healthy individuals, eosinophils are found in the bone marrow, blood, spleen, thymus, gastrointestinal tract, and uterus<sup>67</sup>. Under pathological conditions,

eosinophils can infiltrate other tissues as well. Eosinophils are usually enumerated in the blood because tissue eosinophils are hard to measure. Eosinophil counts over 450 to 500 cells /  $\mu$ l blood are considered mild eosinophilia and counts over 1500 cells / $\mu$ l are characterized as hypereosinophilia<sup>68</sup>.

The main chemotaxins for eosinophils are eotaxins, which homeostatically recruit eosinophils to the gastrointestinal tract, thymus, and uterus<sup>69-71</sup> and to other organs in disease states<sup>72-74</sup>. Humans express three functional eotaxins (CCL11, CCL24, CCL26) whereas mice only express two (CCL11 and CCL24)<sup>75-78</sup>. The eotaxin receptor, CCR3, is highly expressed on eosinophils and to a low level on human basophils, mast cells and Th2 cells (Figure 3)<sup>79, 80</sup>. Other eosinophil chemoattractants include CCL5 and lipid mediators such as leukotriene B4 and prostaglandin D2, although these factors are not specific for eosinophils<sup>81</sup>.

A unique characteristic of eosinophils are their large specific granules, which contain four major granule proteins and numerous cytokines, chemokines, and growth factors (Figure 3)<sup>81</sup>. Cytotoxic effects to host tissues and pathogens have been demonstrated for all major granule proteins: eosinophil cationic protein (ECP), eosinophil-derived neurotoxin (EDN), eosinophil peroxidase (EPX), and major basic protein (MBP)<sup>82</sup>. MBP is highly cytotoxic to mammalian cells, helminths and bacteria due to its disrupting of the cell membrane<sup>83-85</sup>. Other effects of MBP include altering of smooth muscle contraction, inducing mast cell and basophil degranulation, and provoking peripheral nerve plasticity<sup>86-89</sup>. The granule proteins ECP and EDN are ribonucleases<sup>63, 90</sup> with neurotoxic and strong antiviral activities<sup>91, 92</sup> as well as immune modulatory functions<sup>93</sup>. EPX generates reactive oxygen species that are directed extracellularly<sup>94</sup>. These products have cytotoxic, prothrombotic, and proinflammatory effects<sup>94-96</sup>. Granule contents are generally preformed in eosinophils and released upon stimulation. Piecemeal degranulation is the most common process by which eosinophils release their granule contents<sup>97-99</sup>. Specific granule factors, rather than the entire granule, are released in response to an activating signal. This leaves the eosinophil intact and able to respond to subsequent stimulation.



**Figure 3: Cellular structure, receptors and mediators of eosinophils.**

The pseudocolored composite electron micrograph of an eosinophil highlights cellular structures. Characteristic features of eosinophils include the multilobed nucleus, specific eosinophil granules with an electron-dense core and an electron-lucent matrix, lipid bodies, and sombrero vesicles. Eosinophil granules contain cationic proteins, cytokines, growth factors, chemokines and enzymes. The granule contents can be released upon stimulation. Lipid bodies are the place of synthesis for numerous lipid mediators. Granule contents can be released through sombrero vesicles. Eosinophils carry numerous cell surface receptors including chemokine receptors, Fc receptors, pattern recognition receptors, receptors for lipid mediators, cytokine receptors, complement receptors, and adhesion receptors. Abbreviations: 15-HETE, 15-hydroxyeicosatetraenoic acid; APRIL, a proliferation-inducing ligand; CCL, CC-chemokine ligand; CCR, CC-chemokine receptor; CXCL, CXC-chemokine ligand, CXCR, CXC-chemokine receptor; ECP, eosinophil cationic protein; EDN, eosinophil-derived neurotoxin; EGF, epidermal growth factor; EPX, eosinophil peroxidase; GM-CSF, granulocyte-macrophage colony-stimulating factor; IFN, interferon; MBP, major basic protein; NGF, nerve growth factor, PDGF, platelet-derived growth factor; PAF, platelet activating factor; SCF, stem cell factor; TGF, transforming growth factor; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor.



## Chapter 2: Epidemiology of myocarditis and dilated cardiomyopathy

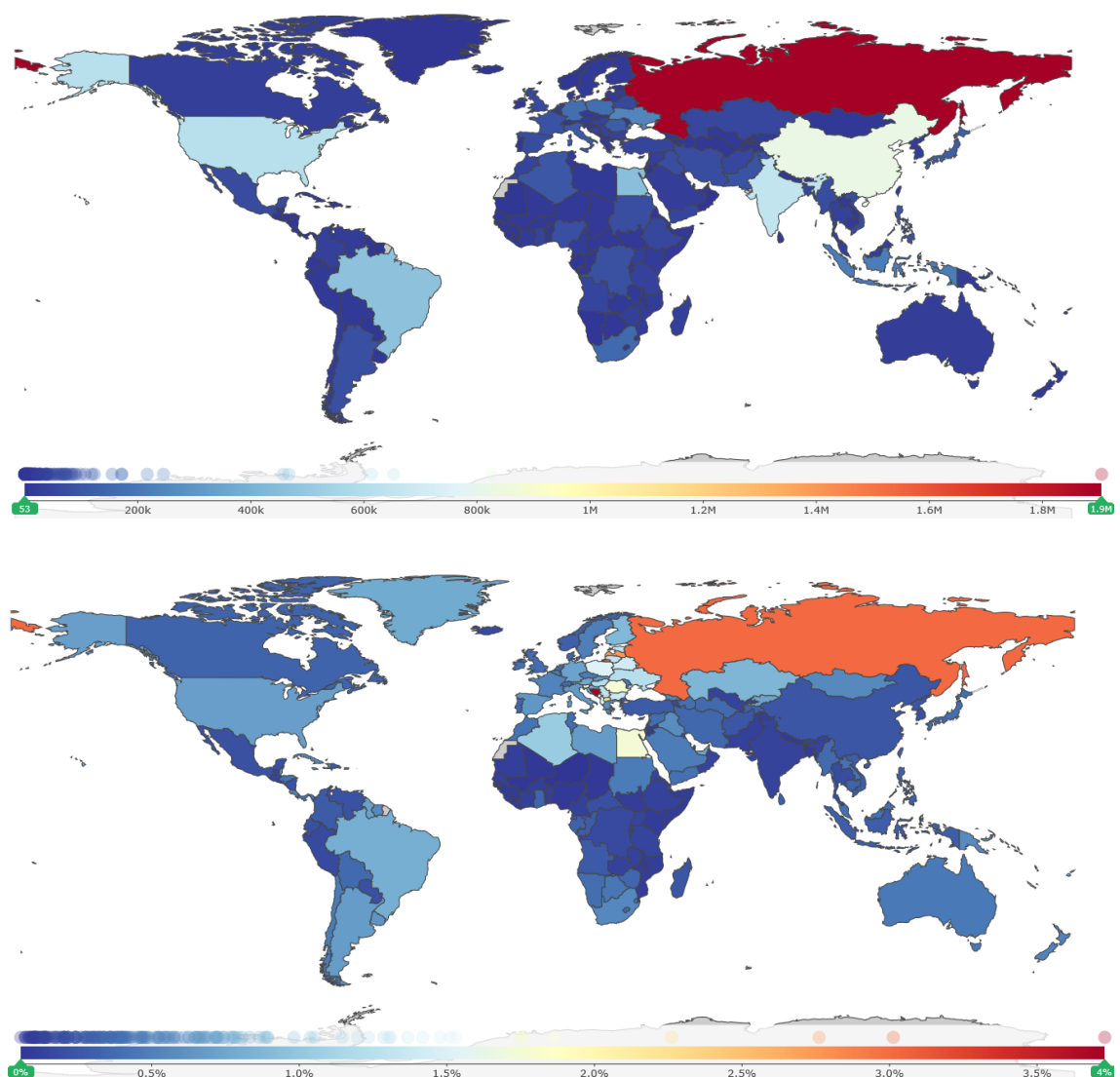
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### Myocarditis

#### Global burden of myocarditis

The writing group on myocarditis for the Global Burden of Diseases, Injuries and Risk Factors 2010 study concluded that accurate, population-based estimates of the incidence or prevalence of myocarditis were not available in any world region<sup>100</sup>. As a result, myocarditis was reported together with cardiomyopathies as “cardiomyopathy and myocarditis” in the Global Burden of Disease 2010 study and still is reported as such in the 2015 study (Figure 4). Cardiomyopathies and myocarditis accounted for 9.2 million or 0.37% of disability-adjusted life years (DALYs) in 2015<sup>101</sup>. The writing group also concluded that myocarditis is responsible for 0.5% to 4% of prevalent heart failure, varying by age and region<sup>100</sup>. A recent review analyzing the cardiovascular diseases of the poorest billion of the world population summarizes causes of heart failure in Sub-Saharan Africa<sup>102</sup>. Cardiomyopathies likely account for about 38% of rural and 22% of urban cases of heart failure in this region. An analysis of the ninth version of the International Classification of Diseases codes estimated the global prevalence of myocarditis to be ≈22 of 100 000 patients annually<sup>103</sup>.

Overall, there is not enough information to compare regional differences in incidence or prevalence of myocarditis or to compare etiologies, risk factors or outcomes with certainty<sup>100</sup>. Endomyocardial biopsies and cardiac magnetic resonance imaging, the diagnostic tests for myocarditis, are not widely available around the world, restricting the ability to identify myocarditis and analyze risk factors and outcomes. Most studies were conducted in North America and Europe and in these countries clinical trials are conducted to test diagnostic strategies and treatments. Given the invasive nature of endomyocardial biopsies, this diagnostic test cannot be employed in large scale or cross-sectional studies. Noninvasive tests are needed to generate better population-based estimates for myocarditis.

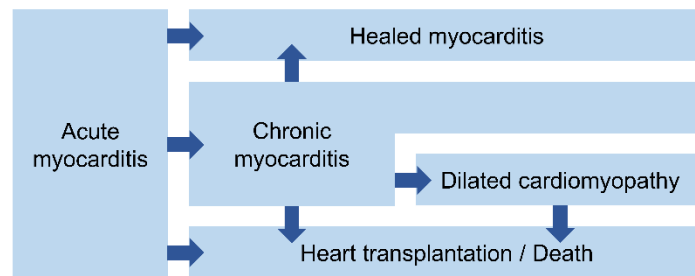


**Figure 4: Estimated disability-adjusted life years lost due to cardiomyopathy and myocarditis in 2015**

Number (top) and percentage of total (bottom) of disability-adjusted life years lost from cardiomyopathy and myocarditis in males and females of all ages by country. Numbers are from the Global Burden of Disease 2015 study<sup>104</sup>.

### Natural history of myocarditis

Due to the heterogeneity of clinical presentations, myocarditis is difficult to diagnose<sup>105</sup>. The gold standard for diagnosis is an endomyocardial biopsy; however, these are not often performed or may have a low sensitivity because of the focal nature of the inflammatory infiltrate<sup>106</sup>. This makes it difficult to identify cases and determine the frequency of the possible outcomes of myocarditis. Some patients with acute myocarditis will recover completely, while others will go on to develop chronic myocarditis or dilated cardiomyopathy (DCM)<sup>2</sup>. Acute myocarditis, both symptomatic and asymptomatic, can also result in sudden cardiac death<sup>107</sup>. Patients who progress to chronic myocarditis / DCM may require heart transplantation or die as a result of the disease later on (Figure 5).



**Figure 5: Possible outcomes of myocarditis.**

Adapted from<sup>2</sup>. The frequency of the different outcomes of acute myocarditis are not exactly known, but estimates suggest that about 60% may recover, about 15-20% go on to develop dilated cardiomyopathy, and the remaining 20-25% will either require a heart transplant or die (for references see text).

Many cases of myocarditis are thought to resolve, yet the exact frequency of patients who heal without sequelae is not known. In a short-term study conducted on pediatric myocarditis patients in the U.S., 62 out of 514 patients (59.6%) had recovered cardiac function by dismissal<sup>108</sup>. This study did not follow up patients beyond hospital dismissal and it is unclear whether recovered cardiac function is equivalent to healed myocarditis. In another study on pediatric myocarditis patients, 71 / 119 patients (60%) achieved normalized cardiac function by 5 years<sup>109</sup>.

In up to 30% of cases, myocarditis can progress to DCM<sup>2</sup>. A review of smaller studies found that on average 21% of myocarditis patients progress to DCM, though the estimates varied widely<sup>110</sup>. In children with myocarditis about 14% had persistent functional abnormalities over a 3.1 year median follow-up period<sup>109</sup>. Importantly, many episodes of myocarditis are subclinical and thus go undiagnosed, making it difficult to estimate the proportion of myocarditis patients that progress to DCM<sup>106</sup>. In contrast, the frequency of myocarditis as a cause of DCM has been addressed in large studies. In a study at Johns Hopkins with over 1,200 DCM patients seen between 1982 and 1998, myocarditis was identified as the cause in 9.2%<sup>111</sup>. In children diagnosed with DCM between 1995 and 2003 from the US and Canada, DCM was caused by myocarditis in 15.6%<sup>112</sup>. In both of these studies, no cause was identified in about 50% of the DCM patients, suggesting that myocarditis could account for an even higher proportion of DCM cases.

Myocarditis can result in sudden death or progress to chronic myocarditis and heart failure, which may lead to death later on. In an Italian study on 174 adult myocarditis patients (mean age 36±18 years) with biopsy-proven myocarditis, transplant-free survival was 74% at 6 years<sup>10</sup>. A smaller study from Korea with 54 patients with biopsy-proven myocarditis reported all-cause mortality of 20.4% after a median follow up of 10.4 years (mean 9.7±5/7 years)<sup>16</sup>. A U.S. study on 112 myocarditis patients (mean age 47.3±15 years) from 1978 to 2002 stated a transplant-free survival rate of 56% at 5 years<sup>113</sup>. For pediatric myocarditis patients in the U.S., transplant-free survival was 74% by 5 years (88 /119 patients)<sup>109</sup>. A large fraction of the deaths/heart transplantations likely happen early after myocarditis diagnosis in children. A retrospective analysis of 514 pediatric acute myocarditis cases from 2006-2011 in the U.S. with a median length of hospital stay of 7 days (IQR: 3-19 days) showed that 21 (4.1%) received heart transplantation and 37 (7.2%) had died at the time of discharge<sup>108</sup>.

Taken together, these studies suggest that of those patients diagnosed with myocarditis, about 60% may recover, about 15-20% go on to develop DCM, and the remaining 20-25% will either require a heart

transplant or die. These proportions may vary by age, sex, ethnicity or location, but sufficient data are unavailable to evaluate these differences.

### **Fatal myocarditis**

Given the wide range of clinical presentations of myocarditis, it is thought to often go undiagnosed or mistaken for other, more common cardiac diseases. Therefore, autopsy series offer a unique possibility of assessing the frequency of myocarditis in deceased individuals. An unselected autopsy series from Italy reports 31 cases (5.1%) of histological myocarditis out of 605 necropsies performed<sup>114</sup>. The highest rate of myocarditis was found in 30-49-year-olds. Interestingly, none of these cases were suspected clinically. The subtype of myocarditis was also determined: 11 cases of lymphocytic myocarditis, 16 cases with granulocytic infiltrate, and 4 other cases. Another single-center, unselected autopsy series from the UK reported the frequency of myocarditis in children 0-18 years of age<sup>107</sup>. Over a 10-year period 1516 autopsies were performed and 28 cases of histological myocarditis (1.8%) were found. The majority of cases were detected in children under 1 year of age, but, as a proportion of deaths, myocarditis was more common in older children (5-8% of deaths in children 5-18 years of age). The majority of children with histological myocarditis died sudden deaths either without any preceding symptoms or with non-specific symptoms that were not recognized as life-threatening. Only 2 of the 28 cases had clinical features of DCM. All cases were classified as lymphocytic myocarditis. These 2 series highlight the discrepancy between lifetime diagnosis of myocarditis and frequency of histological myocarditis at death. Given the lack of reliable epidemiological data on the incidence of myocarditis, it is not possible to compare the incidence with the frequency of myocarditis at death or to estimate the mortality rate for myocarditis. However, these autopsy series show that myocarditis is not an uncommon finding in deceased children and young adults, particular among those who die sudden unexpected deaths. Whether or not myocarditis was the primary cause of death in these individuals cannot be determined with certainty, and it is possible that at least in some cases,

myocarditis was an incidental finding and did not contribute to death. In those who died of sudden deaths, myocarditis is more likely to have been the cause of death.

Several other studies have analyzed cases of sudden death or sudden cardiac death for the presence of myocarditis. Thus, they were preselected on the circumstances of death, in contrast to the above-mentioned autopsy series which were unselected. Recently, a large autopsy series of adult cases was conducted in China<sup>115</sup>. Among 14,487 autopsies, 553 cases of sudden cardiac death were identified and analyzed for the presence of myocarditis by histology. Myocarditis was present in 82 cases (14.8% of sudden cardiac death cases). This would correspond to 0.6% of all autopsies, but only those identified as sudden cardiac death cases were screened for the presence of myocarditis. An additional 14 cases (2.5% of sudden cardiac death cases) were identified as DCM. Myocarditis was more frequent in the young: 35% of 18-34-year-olds, 8% of 35-54-year-olds, and 3% of 55-80-year-olds. DCM was also detected more frequently in younger adults: 6-7% in 18-54-year-olds and less than 1% among 55-80-year-olds. In an autopsy series conducted in Sydney between 1994-2002, autopsies of children and young adults (age 0-35 years) were analyzed<sup>116</sup>. Out of 2986 autopsies, 193 were classified as sudden cardiac deaths. Among those, 23 cases of myocarditis (12%) were detected. This would correspond to 0.77% of all autopsies, but only those identified as sudden cardiac death cases were screened for the presence of myocarditis. Another study determined the frequency of myocarditis in non-traumatic sudden deaths among U.S. military recruits<sup>117</sup>. Over 6 million men and women age 18-35 were monitored and 126 non-traumatic sudden deaths were recorded. Thirteen cases (10%) were due to myocarditis. This would correspond to an incidence rate of 1.3/100,000 person-years, though only those identified as sudden non-traumatic deaths were screened for myocarditis. Importantly, military recruits had already passed a physical examination and medical history screening. An autopsy series in the State of Maryland published this year reviewed all sudden unexpected deaths from January 2005 to December 2014<sup>118</sup>. Out of 62,130 sudden unexpected deaths, 14,733 were autopsied. Of these, myocarditis was determined to be the primary cause of death in 103 cases (0.70%). Myocarditis

subtypes were as follows: lymphocytic myocarditis (56, 54%), neutrophilic (23, 22%), eosinophilic (13, 12.6%), giant cell (2, 1.9%). The mean age at death was 31±17 years and the median age of death was 30 years. Forty-one (40%) of myocarditis cases also had ventricular dilation. In the State of Indiana from 1987 to 1991 the frequency of myocarditis in autopsies was 0.6%<sup>119</sup> and in the State of Michigan it was reported to be 1.3% in the years from 1996 to 2004<sup>120</sup>. Based on death-certificate records from Finland, the incidence rate of fatal myocarditis is 0.46 / 100,000 person-years or 639 cases (0.047%) of 1.35 million deaths between 1970 and 1998<sup>121</sup>. Confirming other studies, the authors found that the proportion of deaths caused by myocarditis was highest in children and adults under 45 years of age (up to 0.64% of deaths in 1-4-year-olds). This study is unique in that it provides a frequency of myocarditis of all deaths, while most other studies have already preselected on sudden or cardiac deaths. However, the sensitivity and specificity of death-records for myocarditis as a cause of death has not been established.

Overall, these studies show very similar frequencies of myocarditis as a proportion of sudden deaths, ranging from 10.0% to 14.8% in children and young adults. The estimates for myocarditis as a frequency of all autopsied deaths in children and young adults varies more widely, from 0.6% to 5.1%, although most estimates are closer to under 1%. Both the percentage of fatal myocarditis as a proportion of total deaths as well as the absolute number of recorded fatal myocarditis cases are highest in children and young adults. Most studies show that in this group, myocarditis may be responsible for close to 1% of deaths.

### **Sex and age differences**

Both myocarditis and DCM are more frequently diagnosed in males than in females<sup>122</sup>. The female / male ratio in myocarditis trials and cohorts was reported between 1:1.5 and 1:1.7<sup>10, 113, 123</sup>. Men are also at a higher risk of fatal myocarditis than women. In a study conducted in Finland, the odds ratio of fatal myocarditis was 1.34 (95% CI: 1.15-1.58, p<0.001) for men compared to women<sup>121</sup>. In a study on recent-onset DCM and myocarditis, transplant-free survival was better in women than in men (4-year transplant-free survival of 96% versus 84%, p=0.003) over a mean follow up of 2.2±1.4 years<sup>124</sup>. In children with

myocarditis, sex was not associated with mortality<sup>108</sup>. Improvement of cardiac function is also less likely for males than for females. Recovery of cardiac function in patients with recent-onset idiopathic DCM or myocarditis with left ventricular ejection fraction under 40% after 6 months was significantly better for women than for men 34% versus 20%,  $p=0.004$ )<sup>124</sup>. Animal models show similar effects of sex on myocarditis severity. Male BALB/c mice have increased CVB3-induced myocarditis<sup>122</sup> and increased severity in experimental autoimmune myocarditis.

There is a strong association of myocarditis with age. Among pediatric cases with acute myocarditis, the number of cases peaks in infancy and in mid-teenage years<sup>108</sup>. Mortality rates are higher in younger children compared to older children<sup>108</sup> and most fatal myocarditis cases are detected in infants<sup>107, 121</sup>. Another peak of myocarditis is in young adults<sup>114, 115, 118, 121</sup>.

### Chagas

Chagas disease is one of the most common causes of non-ischemic cardiomyopathy in Central and South America. Five to eighteen million people are infected with the protozoan *Trypanosoma cruzi*, the causative agent, resulting in over 10,000 deaths per year<sup>125</sup>. Chagas disease predominantly affects the poor living in rural areas<sup>102</sup>. Due to migration, it now also affects over 300,000 infected individuals in the United States<sup>126</sup>.

Chagas disease occurs in 3 phases<sup>125</sup>. The acute phase is typically asymptomatic but can cause fever and malaise in a small proportion of infected individuals. The intermediate phase is asymptomatic and over 50% of individuals will remain in this phase. About 20-30% will progress to chronic cardiovascular Chagas disease (or Chagas cardiomyopathy) after over a decade and others may develop chronic digestive sequela<sup>127</sup>. The clinical manifestations of cardiovascular Chagas disease include left ventricular dilation, myocarditis, congestive heart failure, conduction abnormalities, ventricular arrhythmias, thromboembolic disease and sudden cardiac death<sup>126</sup>. Mortality from Chagas disease is predominantly due to cardiac involvement.



### **Myocarditis as a rare complication in common infectious diseases and vaccines**

Myocarditis is frequently caused by viral infections, particularly from the genus of enteroviruses (eg. Coxsackievirus B3, Parvovirus B19)<sup>13</sup>. In addition, myocarditis can be observed as a rare complication of common viral infections. In an autopsy series on 51 AIDS patients from Mexico, myocarditis was found in 5, pericarditis in 7, and endocarditis in 6 cases<sup>128</sup>. HIV-associated heart failure is common among people living with HIV<sup>129</sup>. In the pre-antiretroviral therapy era, HIV-associated heart failure was related to severe immunocompromise and had a median survival of about 100 days after diagnosis<sup>130</sup>. Today, there are several proposed hypotheses for HIV associated heart failure: direct myocardial damage through HIV infection of cardiomyocytes (which has been shown in vitro), mitochondrial damage in cardiomyocytes, opportunistic infections (due to immune compromise) that cause myocarditis, micronutrient deficiencies (particularly selenium), toxicity of antiretroviral therapy, or autoimmunity<sup>129</sup>. In the era of antiretroviral therapy, HIV infection continues to increase heart failure mortality<sup>131</sup>. The Heart of Soweto study revealed that patients with HIV-related cardiomyopathy had higher viral load and lower CD4<sup>+</sup> T cell counts than people living with HIV without heart failure<sup>131</sup>.

Myocarditis has also been associated with dengue virus infections. In a Colombian study of 102 children with dengue, 11 children (13%) showed symptoms of myocarditis and 1 fatal case showed widespread infection of the heart with dengue<sup>132</sup>. Myocarditis can also be a rare complication of mumps<sup>133</sup> and was associated with the H1N1 influenza epidemic in 2009 in Japan<sup>134</sup>. In a prospective study on recipients of the smallpox vaccine, 10% were found to have new-onset chest pain, dyspnea or palpitations and 2.7% were found to have possible subclinical myocarditis within 30 days of vaccination<sup>135</sup>. In addition to the viral infections commonly associated with myocarditis, other infections or vaccines can cause myocarditis on rare occasions.

### **Lymphocytic myocarditis**

The most common histopathological form of myocarditis is lymphocytic myocarditis. As a result, most of the findings discussed above are either exclusively on lymphocytic myocarditis or, if the subtype was not defined, they are to a large part based on patients with lymphocytic myocarditis. Since the focus of this dissertation is on eosinophils in myocarditis, lymphocytic myocarditis will only be discussed briefly.

Viral infections and autoimmune reactions are the most common causes of lymphocytic myocarditis (see Chapter 1). Among patients with acute lymphocytic myocarditis, those with circulating autoantibodies against cardiac antigens are most likely to benefit from immunosuppressive treatment (improvement of left ventricular ejection fraction), while those with viral genomes in the heart are likely not to respond<sup>136, 137</sup>. This highlights how the role of the immune system in myocarditis varies depending on the etiology of the disease.

A large study from Australia on childhood DCM analyzed the frequency of lymphocytic myocarditis<sup>138</sup>. Frequency of lymphocytic myocarditis in pediatric DCM was 35%. Six of 9 cases who presented with sudden death (33%) had lymphocytic myocarditis. For those who underwent endomyocardial biopsy in the first two months after presentation, the rate of lymphocytic myocarditis was even higher at 40%<sup>139</sup>. Despite this high initial mortality, long-term survival was better for children with lymphocytic myocarditis than for those with nonspecific histological findings<sup>138</sup>. The likelihood of survival was also addressed in a large single-center series of biopsy-proven myocarditis from Massachusetts General Hospital<sup>113</sup>. Of 112 consecutive patients with histopathologic confirmation of myocarditis, 88 (79%) were alive without cardiac transplantation at 1 year and 63 (56%) at 5 years. Among those with lymphocytic myocarditis (55%) transplant-free survival for patients was 80% at 1 year, 50% at 5 years and, 45% at 10 years. Compared to patients with borderline myocarditis, survival was much worse for patients with lymphocytic myocarditis. In contrast to the studies above from Australia, this population included adults. The mean age was 47±15.7 years. A large case series of patients undergoing heart transplantation looked at the prognosis for patients with lymphocytic

myocarditis in the explanted hearts<sup>140</sup>. Out of 32 patients with histological evidence of lymphocytic myocarditis in the explanted heart, only 6 (19%) had been clinically diagnosed with myocarditis. The majority (20 patients, 63%) had been diagnosed with idiopathic DCM. The prognosis for patients with lymphocytic myocarditis in explanted hearts was compared to age- and gender-matched controls with either idiopathic DCM or ischemic cardiomyopathy (based on clinical diagnosis). Survival post transplantation was not significantly different between the 3 patient groups. The 5-year survival rate post transplantation for lymphocytic myocarditis patients was 86% in this study. However, lymphocytic myocarditis patients had a higher frequency of acute cellular rejections compared to those with ischemic cardiomyopathy. More studies will be needed to establish the prognosis for patients with heart transplantations based on the etiology of the underlying cardiomyopathy.

### **Eosinophilic myocarditis**

Endomyocardial biopsies of myocarditis patients are often not performed, or the histopathological type of myocarditis is not reported. Therefore, it is very difficult to identify the subtype of myocarditis and the prevalence or incidence of eosinophilic myocarditis cannot be estimated. In studies where histopathological types are reported, eosinophilic myocarditis varies from 2% - 22.6% of all myocarditis cases<sup>16, 113, 118, 141</sup>. In a large study in Maryland, eosinophilic myocarditis accounted for 12.6% of all fatal myocarditis cases<sup>118</sup>. Eosinophilic myocarditis is less common than lymphocytic myocarditis but more frequent than giant cell myocarditis. It is unclear whether there are age or gender differences among eosinophilic myocarditis patients. Eosinophilic myocarditis has numerous possible etiologies (see Chapter 1, Table 3), many of which likely vary in prevalence across different regions. To date, no studies have addressed these geographic variations.

### **Mortality in eosinophilic myocarditis**

It is not known what proportion of eosinophilic myocarditis patients will require heart transplantations or die from this disease. A compilation of several studies with at least 3 biopsy-proven eosinophilic myocarditis patients yields an overall transplant-free survival rate of 90% (Table 5). However, many studies did not report the time of follow-up and the underlying causes may not be representative of the true distribution.

A recent study from Korea suggests that patients with eosinophilic myocarditis may fare better than lymphocytic myocarditis patients<sup>16</sup>. Of 54 patients with biopsy-proven myocarditis, 29.6% were lymphocytic, 22.2% eosinophilic, and 48.1% borderline myocarditis. Underlying causes of eosinophilic myocarditis were not assessed. The median follow up for all patients was 10.4 years. Five (42%) of lymphocytic myocarditis patients died but none of the eosinophilic myocarditis patients. A study from Japan reported on 7 patients with eosinophilic myocarditis identified among 64 patients admitted with eosinophilia over a 27-year period<sup>142</sup>. The etiologies were identified as follows: 3 idiopathic, 2 eosinophilic granulomatosis with polyangiitis (EGPA), 1 parasitic infection (*Toxocara canis*), 1 chronic eosinophilic leukemia (hypereosinophilic syndrome, HES). Six out of 7 patients were alive at the end of the study with a mean survival time of 37 ±40 months. Another Japanese case series excluded patients with EGPA, HES, or allergic myocarditis (although the criteria used for diagnosis were not clear), leaving only patients with idiopathic eosinophilic myocarditis (n=22) and lymphocytic myocarditis (n=7)<sup>143</sup>. None of the patients had died one year after discharge. In a relatively large study with 112 myocarditis patients (62 lymphocytic, 25 borderline, 11 granulomatous, 7 eosinophilic, 7 giant cell), six patients underwent cardiac transplantation within the first year<sup>113</sup>. Of these six, four had eosinophilic myocarditis, 1 had giant cell myocarditis, and 1 had granulomatous myocarditis. Thus, 4/7 eosinophilic myocarditis patients required heart transplantation within one year of diagnosis. These studies report vastly different transplant-free survival rates for eosinophilic myocarditis, from 100% over 10 years to less than 50% in one year, making it impossible to draw conclusions on the expected survival rate. Several factors like treatments strategies, underlying

etiology, additional co-morbidities, or patient characteristics may explain the differences between studies and should be explored further.

**Table 5: Reported mortality in eosinophilic myocarditis**

| Reference                                | n   | Etiology (#)   | Follow-up time<br>(years, mean±SD) | Transplant-free<br>survival<br>(survived / total) |
|--|-----|--|------------------------------------|---|
| Sohn et.al., 2015 <sup>146</sup>         | 4   | HES (2)<br>Parasitic (2)   | n.a.                               | 4/4   |
| Youn et.al., 2014 <sup>16</sup>          | 12  | n.a.   | 10±4                               | 12/12   |
| Comarmond et.al., 2013 <sup>32</sup>     | 63  | EGPA (63)  | 5                                  | 58/63   |
| Kawano et.al., 2011 <sup>142</sup>       | 7   | EGPA (2)<br>HES (1)<br>Idiopathic (3)<br>Parasitic (1)   | n.a.                               | 6/7   |
| Yanigasawa et.al., 2011 <sup>143</sup>   | 22  | Idiopathic (22)  | 1                                  | 22/22   |
| Al Ali et.al., 2006 <sup>147</sup>       | 3   | Hypersensitivity (1)<br>Idiopathic (2)   | n.a.                               | 3/3   |
| Magnani et. al., 2006 <sup>113</sup>     | 7   | n.a.   | 1                                  | 3/7   |
| Starza et.al., 2005 <sup>148</sup>       | 4   | HES (4)  | 4.9±8 (4)                          | 4/4   |
| Vandenberghe et.al., 2004 <sup>149</sup> | 3   | HES (3)  | 3.8±1.1                            | 2/3   |
| Arima et.al., 2002 <sup>150</sup>        | 5   | n.a.   | n.a.                               | 4/5   |
| Vargo et.al., 1977 <sup>151</sup>        | 3   | Parasitic (3)  | n.a.                               | 1/3   |
| Total                                    | 133 | EGPA (65)<br>HES (10)<br>Hypersensitivity (1)<br>Idiopathic (27)<br>Parasitic (5)<br>n.a. (24) | n.a.                               | 120/133<br>(90.2%)                                |

Abbreviations: HES, hypereosinophilic syndrome; EGPA, eosinophilic granulomatosis with polyangiitis; n.a., not available.

### **Hypersensitivity myocarditis**

Numerous drugs have been reported as the potential cause of hypersensitivity myocarditis (Table 3). The frequency of this complication has only been reported for a few drugs, such as clozapine. An incidence of 0.7-1.2% has been reported for clozapine-induced myocarditis<sup>144</sup>. Symptoms start around 3 weeks from drug initiation. Out of those patients, 10% died, about half recovered and 15% showed persistent left ventricular dysfunction. For the remaining 25% no follow-up information was available<sup>145</sup>. Drug rash with eosinophilia and systemic symptoms (DRESS) is a systemic hypersensitivity reaction to drugs with a 5-10% fatality rate<sup>29</sup>. In a review of 22 reported cases with myocarditis, 12 (55%) died, another 2 (9%) had continued cardiac insufficiency and the remaining patients (36%) recovered<sup>29</sup>. Hypersensitivity myocarditis is a rare but life-threatening complication of numerous drugs. For most agents, the frequency of this complication is unknown and the causative agent cannot always be identified unequivocally. More research is required to identify patients who may be at risk of this side effect.

### **Hypersensitivity myocarditis in heart transplantations**

In heart transplant patients, findings of eosinophilic myocarditis in the explanted heart are not uncommon<sup>152-155</sup>. This is thought to be a hypersensitivity reaction to drugs as patients awaiting heart transplants usually take a multitude of drugs to sustain them until a donor is found. The first report on eosinophilic myocarditis in a large cohort of transplant patients was published in 1991<sup>152</sup>. Among 193 heart transplant patients, 15 (7%, 13 male, 2 female) had eosinophilic myocarditis. Interestingly, none of these were detected clinically, which the authors attribute to the already severe cardiac conditions of these patients, making it difficult to observe a worsening in heart function. Another study, however, did report worsening of cardiac function in 3 patients<sup>156</sup>. Of the 15 cases, 14 developed eosinophilia within 2 weeks

prior to transplant<sup>152</sup>. All cases had received multiple drugs, which were thought to have provoked hypersensitivity myocarditis. Another 2 recent reports on 111 and 190 transplant patients found a very similar proportion with hypersensitivity myocarditis (8 cases, 7.2% and 14 cases, 7.4%) in explanted hearts<sup>153, 154</sup>. Of these, 4/8 (50%) and 12/14 (86%) had eosinophilia before transplantation. Most pre- and post-transplant characteristics were similar between patients with and without hypersensitivity myocarditis and there was no difference in post-transplant survival in patients with or without eosinophilic myocarditis. None of the 14 patients showed recurrent eosinophilic myocarditis on post-transplant biopsies<sup>154</sup>. Multiple drug therapy, and specifically inotropic support was suspected to have caused the hypersensitivity reaction leading to eosinophilic myocarditis. The most recent and largest study on 759 heart transplant patients showed a lower rate of eosinophilic myocarditis with only 21 cases (2.7%)<sup>155</sup>. The authors noted a potential trend to decreasing hypersensitivity myocarditis from 2000 to 2010. Again, none of the cases were diagnosed clinically and post-transplant survival was similar in those with and without eosinophilic myocarditis. There was, however, increased acute cellular reaction in the transplanted hearts of those patients with hypersensitivity myocarditis in the explanted heart compared to other transplants. About 4,000 to 5,000 heart transplantations are performed annually worldwide, with the vast majority taking place in North America and Europe<sup>157</sup>. At a rate of 2.7% to 7.4% of hypersensitivity myocarditis, an estimated 108-370 cases of hypersensitivity myocarditis in patients awaiting heart transplantation likely occur worldwide per year.

### **Tropical endomyocardial fibrosis**

Tropical endomyocardial fibrosis is a form of eosinophilic endomyocarditis that is concentrated in Sub-Saharan Africa, particularly Uganda, Kenya, Tanzania, Mozambique, Gabon, Congo, Cameroon, Sudan, Nigeria, Cote d'Ivoire and Ghana<sup>158</sup>. In addition, it can be found in parts of South America and Asia<sup>38</sup>. The etiology of endomyocardial fibrosis is not known. One hypothesis is that helminth-induced chronic eosinophilia leads to endomyocardial fibrosis in a process similar to that in patients with hypereosinophilic

syndrome<sup>159</sup>. Others believe that there is a toxic cause<sup>160</sup>. In endemic regions, tropical endomyocardial fibrosis may account for a large proportion of heart disease (20% of cases referred for echocardiography in a study in Uganda)<sup>161</sup> and the disease is most frequent in children and young adults<sup>158</sup>. Mortality is very high with 25% survival by 2 years in a study from the 1970s<sup>162</sup>. More recent estimates suggest that tropical endomyocardial fibrosis may account for 1% of heart failure in Sub-Saharan Africa<sup>102</sup>. Eosinophilia has been reported as a risk factor in Uganda and Nigeria, and duration of disease is inversely correlated with eosinophilia<sup>158</sup>, suggesting that eosinophils may be involved in the initiation or early stages of disease.

### **Eosinophilic granulomatosis with polyangiitis**

Eosinophilic granulomatosis with polyangiitis (EGPA) is one of the rarest systemic necrotizing vasculitides. The incidence is estimated to be approximately 0.11 to 2.66 cases per 1,000,000 person-years and the prevalence is about 10.7 to 14 per 1 million adults<sup>163</sup>. A total of 383 patients have been enrolled in the French Vasculitis Study Group cohort since 1983<sup>32</sup>. Among these patients, the 5-year survival rate is 90%. For patients with cardiac involvement, the 5-year survival rate is substantially lower at 78%. Eosinophilic myocarditis develops in about one third of EGPA patients<sup>164</sup>. While EGPA is an extremely rare disease, a large proportion of patients (one third) develops eosinophilic myocarditis, which contributes to mortality.

### **Hypereosinophilic syndrome**

Hypereosinophilic syndrome is a rare and complex disease defined by prolonged hypereosinophilia (> 1500 / $\mu$ l blood) and organ damage in the absence of other primary causes<sup>35</sup>. The largest report on HES patients was a multicenter study from 11 institutions in the U.S. and Europe with 188 patients<sup>165</sup>. Cardiac manifestations of HES include necrotizing myocarditis, mural thrombus formation and fibrosis, particularly of the endocardium<sup>166</sup>. These occur in 5%-58% of HES patients<sup>35</sup>. The survival rate for patients with HES is not clear, but cardiac manifestations are thought to be the major contributor to morbidity and mortality<sup>33</sup>.



### **Giant cell myocarditis**

Giant cell myocarditis is the rarest form of myocarditis<sup>118</sup>. The diagnosis requires endomyocardial biopsies with myocyte injury with or without necrosis<sup>167</sup>. The key characteristic is the presence of multinucleated giant cells forming part of the inflammatory infiltrate with lymphocytes and eosinophils. As eosinophils are almost always a dominant feature of giant cell myocarditis, this disease is briefly discussed here. About one third of patients has moderate eosinophil infiltration<sup>167</sup>. Giant cell myocarditis is thought to be autoimmune in nature; 17-19% of patients suffer from additional non-cardiac autoimmune diseases<sup>167-169</sup>. For Finland, the incidence rate of giant cell myocarditis was estimated as 0.67 per 100,000 person-years in the adult (> 18 years of age) population<sup>167</sup>. No other data on the incidence of this type of myocarditis exist. However, the frequency of giant cell myocarditis in autopsies was evaluated in India. Autopsy records from a tertiary referral center revealed 12 cases of giant cell myocarditis (0.005% of autopsies, 0.8% of myocarditis cases) between 1994 to 2010<sup>170</sup>. Giant cell myocarditis is thought to be the most severe form of myocarditis, frequently resulting in death or heart transplantation. In an earlier study, the median survival was 5.5 months from the onset of symptoms. More recently, median survival was reported to be around 5 years<sup>167, 171</sup>. Strong immunosuppressive treatment may improve survival<sup>169, 171</sup>. Whether eosinophils contribute to mortality in giant cell myocarditis remains to be determined.

In summary, eosinophilic myocarditis is a relatively rare form of myocarditis, accounting for about 2-22% of myocarditis patients. Eosinophilic myocarditis is more frequently found in certain groups, such as EGPA or HES patients and those awaiting heart transplantations. In addition, eosinophilic myocarditis can occur as a rare complication in patients with parasitic infections or as part of a hypersensitivity reaction to drugs.

### **Dilated cardiomyopathy**

DCM is defined as left ventricular or biventricular systolic dysfunction (abnormal ejection fraction) and dilation (> 2 standard deviations from normal) that are not explained by abnormal loading conditions or

coronary artery disease<sup>47</sup>. The incidence rate for DCM in adults is about 5-8 / 100,000 person-years and the prevalence is 36-40 / 100,000 adults<sup>172</sup>. DCM studies report a female/male ratio between 1:1.3 and 1:1.5<sup>122</sup>. DCM is the major cause of heart failure in patients under 40 years of age. The 5-year survival rate is less than 50%<sup>173</sup>. About 9-16% of patients with new onset DCM have evidence of prior myocarditis<sup>48-50</sup>. Myocarditis as a cause of DCM was associated with poor survival post transplantation<sup>174</sup>.

### **Dilated cardiomyopathy in children**

In children, DCM is the most common cardiomyopathy and the leading indication for heart transplantation<sup>175</sup>. The presenting symptom of DCM in children is often heart failure<sup>176</sup>. The incidence of pediatric DCM is 0.57-0.76 / 100,000 person-years<sup>139, 177, 178</sup> and is higher in younger children compared to older children<sup>139</sup>. Myocarditis may cause a higher proportion of DCM in children than in adults. Myocarditis was identified as the cause of DCM in 16% of pediatric patients in one study<sup>176</sup> and in 40% of patients biopsied within 2 months of diagnosis in another study<sup>139</sup>. Recovery of heart function in children with DCM is possible, but a large proportion will experience death or require heart transplantation. By one year of follow-up, 29-34% of pediatric DCM patients experience either death or heart transplantation<sup>112, 178</sup>. Another study found that by 2 years of follow-up, 22% had recovered LV function and size, 51% had experience death or heart transplantation and 27% lived with persistent functional deficiencies<sup>179</sup>. Longer follow-up studies show that there is an ongoing risk of death. About 40% of children with DCM due to myocarditis and almost 60% of children with idiopathic DCM experienced transplantation or death at 10 years of follow-up<sup>109, 112</sup>. The cost of pediatric heart failure is not entirely known, but the average hospital charge for hospitalization due to heart failure in the US was over US\$ 70,000 in 2009<sup>180</sup>. DCM may disproportionately affect children in disadvantaged populations. In a study conducted in Australia, indigenous Australian children had an increased risk of DCM (relative risk 2.67, 95% confidence interval: 1.42–4.63) and a higher rate of death as presenting symptom<sup>139</sup>. The authors suggest that this may be a result of decreased availability of health care.

DCM is a major cause of heart failure in children and young adults and carries a dire prognosis. Myocarditis accounts for 10-40% of DCM cases and the frequency is likely higher in children than in adults. It is not entirely clear if DCM due to myocarditis carries a more or less favorable outcome compared to other types of DCM.

## **Conclusions**

Large gaps remain in our understanding of the burden of myocarditis. To date, there are no reliable estimates of the incidence or prevalence of myocarditis. Comparisons of regional differences in incidence, etiology, or risk factors are therefore not possible. However, it seems clear that men are at a higher risk of myocarditis than women. Moreover, we have a good understanding of the natural history of myocarditis. About 60% of myocarditis patients may recover, about 15-20% go on to develop DCM, and the remaining 20-25% will either require a heart transplant or die. Most studies on myocarditis and DCM come from North America, Western Europe, Japan, and Australia. It is possible that there are large differences in the epidemiology of myocarditis and DCM in different regions. For example, it has been suggested that cardiomyopathies are endemic in Sub-Saharan Africa<sup>181</sup>. Myocarditis likely cause 10-40% of DCM cases. DCM in turn is the major indication for heart transplantations in children and young adults and a major cause of heart failure. Thus, myocarditis contributes significantly to the burden of heart failure. Our knowledge on the myocarditis subtype of eosinophilic myocarditis is even more limited. No reliable data exist on the incidence / prevalence, natural history, or outcomes of eosinophilic myocarditis. Nevertheless, eosinophilic myocarditis contributes significantly to morbidity and mortality in patients with EGPA and HES, is frequently found in patients awaiting heart transplantation, and is a rare, but potentially fatal complication of commonly used drugs and common infectious diseases. More research is required to begin filling the knowledge gaps in the epidemiology of myocarditis and specifically eosinophilic myocarditis.

## Chapter 3: Eosinophil trafficking to the heart in eosinophilic myocarditis

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Diny NL, Hou X, Barin JG, Chen G, Talor MV, Schaub J, Russell SD, Klingel K, Rose NR, Čiháková D. Macrophages and cardiac fibroblasts are the main producers of eotaxins and regulate eosinophil trafficking to the heart. *Eur J Immunol*. 2016 Dec;46(12):2749-2760. (Copyright Wiley-VCH Verlag GmbH & Co. KGaA. Reproduced with permission.)

### Summary

Cardiac manifestations are a major cause of morbidity and mortality in patients with eosinophil-associated diseases. Eosinophils are thought to play a pathogenic role in the heart. We investigated the pathways that recruit eosinophils to the heart using a model of eosinophilic myocarditis where experimental autoimmune myocarditis is induced in IFN $\gamma$ <sup>-/-</sup>IL-17A<sup>-/-</sup> mice. Two conditions have to be met for efficient eosinophil trafficking to the heart: high eotaxin (CCL11, CCL24) expression in the heart and expression of the eotaxin receptor CCR3 by eosinophils. We identified the source of CCL11 as cardiac fibroblasts with interstitial localization in the heart. CCL24 is produced by F4/80<sup>+</sup> macrophages localized at inflammatory foci. Expression of these chemokines is controlled by Th2 cytokines, IL-4 and IL-13. To determine the relevance of this pathway in humans, we analyzed endomyocardial biopsy samples from myocarditis patients. Expression of CCL11 and CCL26 was significantly increased in eosinophilic myocarditis compared to chronic lymphocytic myocarditis and positively correlated with the number of eosinophils. Thus, eosinophil trafficking to the heart is dependent on the eotaxin-CCR3 pathway in a mouse model and associated with eotaxin expression in patients with eosinophilic myocarditis.

## Introduction

Cardiac manifestations are a major cause of morbidity and mortality in patients with eosinophilia. In hypereosinophilic syndrome (HES), 20-50% of patients develop fibrosis or inflammation of the endocardium, myocardium, or pericardium<sup>33, 34, 182</sup>. Similarly, one third of patients with eosinophilic granulomatosis with polyangiitis (EGPA) suffer from cardiovascular manifestations<sup>32, 35</sup>. Eosinophilic myocarditis can also develop in the absence of prolonged peripheral eosinophilia, for example, in the potentially fatal form of acute necrotizing eosinophilic myocarditis, in patients with parasitic diseases, or as hypersensitivity myocarditis in response to drugs<sup>14, 24</sup>. No specific biomarkers or treatments are currently available for eosinophilic myocarditis; patients usually receive immunosuppressive therapy and symptomatic treatment in later stages<sup>13, 24, 183</sup>. A recent report from the NIH taskforce on the Research Needs of Eosinophil-Associated Diseases underscored the need for animal models to study organ-targeted eosinophil accumulation<sup>184</sup>.

Given the strong association of eosinophils with cardiac disease, eosinophils are believed to play a pathogenic role in the heart<sup>40, 185</sup>. Eosinophils may be directly cytotoxic to cardiomyocytes<sup>39, 43, 44</sup>, activate cardiac mast cells<sup>45</sup>, or release pro-thrombotic tissue factor<sup>46</sup>. Eosinophil granule proteins are deposited in the myocardium during eosinophilic myocarditis<sup>186</sup>. They are thought to damage the endocardium, resulting in thrombosis and endocarditis, and eventually in endomyocardial fibrosis and valvular complications<sup>33, 182</sup>. Animal studies further strengthen the evidence that eosinophils contribute to pathology and mortality in eosinophilic myocarditis<sup>187, 188 189</sup>.

Despite the clear link between eosinophils and cardiac damage, the pathways that recruit eosinophils to the heart have not been described. Eosinophils develop in the bone marrow and are released into the blood in response to IL-5. From there, they migrate into tissues<sup>66</sup>. Eosinophils express numerous receptors for chemokines and other mediators that can direct trafficking<sup>190</sup>. The eotaxins are the strongest eosinophil

attracting chemokines<sup>191</sup> and the most studied<sup>69, 71, 72, 192</sup>. Whether eotaxins play a role in eosinophil trafficking to the heart, however, is not known.

To discover the signals responsible for eosinophil trafficking to the heart, we used a mouse model of eosinophilic myocarditis previously established by our group<sup>189</sup>. Induction of experimental autoimmune myocarditis (EAM) in mice lacking the key Th1 and Th17 cytokines IFN $\gamma$  and IL-17A results in a fatal, Th2-driven form of myocarditis that is characterized by a predominantly eosinophilic infiltrate. We identified the signaling pathway controlling eosinophil trafficking to the inflamed heart and the cell types producing the required chemoattractants. We then validated our findings in patients with eosinophilic myocarditis.

## Methods

### Patients

Endomyocardial biopsies were taken at multiple centers for routine diagnostic purposes to evaluate the pathologic basis of unspecified congestive heart failure. Samples were investigated in the Department of Molecular Pathology at the University Hospital of Tübingen, Germany by histology, immunohistochemistry and molecular pathology to identify infectious agents in the myocardium as described<sup>193</sup>. Written informed consent for further examination was obtained from all patients. We analyzed remaining de-identified tissue from 14 patients with chronic lymphocytic myocarditis and 16 patients with eosinophilic myocarditis. Diagnosis of myocarditis was based on established criteria<sup>2</sup> and all samples were negative for viral infections. Patient age and gender are summarized in Table 6.

**Table 6: Patient characteristics**

| Characteristic               | Chronic myocarditis (n=14) | Eosinophilic myocarditis (n=16) | P value |
|------------------------------|----------------------------|---------------------------------|---------|
| Age in years (median, range) | 54, 42-79                  | 53.5, 29-82                     | 0.557*  |
| Female sex (n, %)            | 3, 21.4%                   | 10, 62.5%                       | 0.024†  |

\* *t*-test

† chi-square test

## Animals

Wildtype (WT, JAX 0651),  $\Delta$ dblGATA1 (JAX 5653<sup>194</sup>), IFN $\gamma$ <sup>-/-</sup> (JAX 2286), and CCR3<sup>-/-</sup> mice (JAX 5440), all on the BALB/c background, were obtained from Jackson Laboratories (Bar Harbor, ME). IL-5 transgenic (IL-5Tg) founder mice<sup>195</sup> were kindly provided by James Lee and Nancy Lee (Mayo Clinic Arizona, Scottsdale, AZ). IL-17A<sup>-/-</sup> founder mice were generously provided by Yoichiro Iwakura (University of Tokyo, Tokyo, Japan). IFN $\gamma$ <sup>-/-</sup> mice were crossed with IL-17A<sup>-/-</sup> mice and bred to homozygosity. Resulting IFN $\gamma$ <sup>-/-</sup>IL-17A<sup>-/-</sup> mice were then crossed with  $\Delta$ dblGATA1 mice or with CCR3<sup>-/-</sup> mice and offspring were intercrossed to generate mice homozygous at all loci. All mice were maintained at the Johns Hopkins University School of Medicine specific pathogen-free animal vivarium. The number of animals used in each experiment and the number of repeats are listed in the figure legends. Experiments were performed in accordance with the guidelines set forth in the Guide for the Care and Use of Laboratory Animals<sup>196</sup>. All methods and protocols were approved by the Animal Care and Use Committee of the Johns Hopkins University.

## Experimental autoimmune myocarditis

To induce myocarditis, male mice age 6-9 weeks were immunized on days 0 and 7 with 100  $\mu$ g MyHC $\alpha$ <sub>614-629</sub> peptide emulsified in Complete Freund's Adjuvant (Sigma-Aldrich) supplemented to 5 mg/ml with heat-killed *Mycobacterium tuberculosis* strain H37Ra (Difco). Mice were also administered 500 ng pertussis toxin (List Biologicals) *ip* on day 0.

## Eosinophil isolation

Blood from female IL-5Tg and CCR3<sup>-/-</sup>IL-5Tg donors was collected into phosphate buffered saline (PBS) with 100 U/ml Heparin, layered over Histopaque-1119 (Sigma-Aldrich), and centrifuged. Interphase cells were collected and washed in PBS. Red blood cells were lysed with ACK buffer (Quality Biologicals). Eosinophils were enriched by negative MACS selection with anti-CD90.2 and anti-CD45R beads (Miltenyi Biotech).

Eosinophil purity was determined by flow cytometry staining for CD11b, Ly6G, Ly6C, and SiglecF and ranged from 87% to 93%.

### **Flow cytometry and cell sorting**

Hearts were perfused through the ventricles with PBS for 3 min. Single-cell suspensions were generated from heart tissue by digestion with Collagenase II and DNase I (Worthington) in HBSS in GentleMACS C Tubes according to manufacturer's instructions (Miltenyi Biotech). Splenocytes were isolated into single-cell suspension, and bone marrow cells were collected from femurs followed by red blood cell lysis with ACK buffer. Intestinal lamina propria cells were isolated from the distal 5 cm of the small intestine. Intestines were flushed, cut into <1 cm pieces, and incubated twice in HBSS, 5% fetal bovine serum (FBS), 5mM EDTA, 10mM HEPES, and Penicillin/Streptomycin for 20 min at 37°C. Supernatants were discarded between incubations. Tissues were incubated for 60 min at 37°C in HBSS with  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ , 10mM HEPES, 5% FBS, 1 mg/ml collagenase D, 0.1 mg/ml DNase I, and 3 mg/ml Dispase II (Roche). Using GentleMACS C Tubes, cells were mechanically separated according to manufacturer's instructions (Miltenyi Biotech). Following filtration through 70 $\mu\text{m}$  filters, single-cell suspensions were stained with LIVE/DEAD stain (Molecular Probes), washed, Fc $\gamma$ RII/III blocked with  $\alpha$ -CD16/CD32, and stained with fluorochrome-conjugated antibodies (eBioscience, BioLegend, BD Pharmingen). For detection of *Ccl11* mRNA, samples were processed using the FlowRNA II Assay kit (Affymetrix eBioscience) according to manufacturer's protocols. In brief, samples were stained with fluorochrome-conjugated antibodies against surface antigens, fixed and permeabilized, and hybridized with a type 1 *Ccl11* probe or *Actb* control probe. Signal amplification was achieved through a branched DNA structure, which was then hybridized to a probe conjugated to Alexa Fluor647. Samples were acquired on a LSRII 4-laser cytometer running FACSDiva 6.0 (BD Immunocytometry) and analyzed using FlowJo 10.0.8 (TreeStar Software). For cell sorting, single cell suspensions were layered over a Histopaque-1119 gradient, stained, and sorted on an Arianu cell sorter.



### **Cardiac fibroblast isolation and culture**

Primary adult mouse cardiac fibroblasts were isolated as described<sup>197</sup>. Hearts were cannulated through the aorta and perfused for 3 min at 37°C and 4 ml/min with perfusion buffer: 7.03g/L NaCl, 1.1 g/L KCl, 0.082 g/L KH<sub>2</sub>PO<sub>4</sub>, 0.085 g/L Na<sub>2</sub>HPO<sub>4</sub>, 0.144 g/L MgSO<sub>4</sub>, 2.38 g/L HEPES, 0.39 g/L NaHCO<sub>3</sub>, 1 g/L glucose, 3.74 g/L Taurine, 2 g/L 2,3-Butanedione monoxime (all Sigma). Next, hearts were perfused for 4 min with perfusion buffer supplemented with 12 g/L Collagenase II (Worthington) and 0.5 g/L Protease XIV (Sigma-Aldrich), and for 8 min with addition of digestion enzymes and 0.03M CaCl<sub>2</sub>. Hearts were cut into small pieces and cells separated by repeated pipetting for 3 min. Cells were filtered through a 70 µm filter and washed in DMEM (Gibco). Cells were plated in DMEM with 20% FBS (GE Healthcare Life Sciences), nonessential amino acids (Sigma), Penicillin/Streptomycin, 2mM L-Glutamine, and 25mM HEPES (all Quality Biological). Non-adherent cells were washed away after 1h. Fibroblasts of passage 2-3 were used in experiments. Cells and supernatants were harvested at the indicated time points after addition of recombinant mouse cytokines (R&D Systems).

### **Bone marrow-derived macrophage culture**

Bone marrow was isolated from adult WT male mice, red blood cells lysed with ACK buffer, and cells cultured in complete DMEM as above with 20 ng/ml recombinant mouse M-CSF and 10 ng/ml GM-CSF (R&D Systems) for 10 days. Media was replaced on days 3 and 6. On day 10, cells were seeded in media without M-CSF or GM-CSF. Cells and supernatants were harvested 24h after stimulation with cytokines on day 11 (R&D Systems).

### **Quantitative Real-time PCR**

Mouse tissue and cell mRNA was extracted in TRIzol (Invitrogen), quantitated by spectrophotometry, reverse transcribed (High-Capacity cDNA Reverse Transcription Kit, Applied Biosystems), and amplified with Power SYBR Green PCR Master Mix (Bio-Rad) on a MyiQ2 thermocycler running iQ5 software (Bio-Rad). Primers for mouse genes (*Gapdh*: 5'-TCCTCCTCAGACCGCTTTT-3' and 5'-TCTGCTGGAGTCCCCTTG-3',

*Ccl11*: 5'-GAATCACCAACAACAGATGCAC-3' and 5'-TCCTGGACCCACTTCTTCTT-3', *Ccl24*: 5'-TCTTAGGGCCCTTCTTGGTG-3' and 5'-AATTCCAGAAAACCGAGTGG-3') were commercially synthesized (Integrated DNA Technologies). Data were analyzed using the  $2^{-\Delta\Delta C_t}$  method<sup>198</sup> by normalizing threshold cycles to Glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) and controls. RNA from endomyocardial biopsies was isolated using the PureLink FFPE RNA Isolation Kit (Invitrogen) from three to five 5- $\mu$ m-thick sections per sample, reverse transcribed, and amplified using the iTaq Universal Probes Supermix (Bio-Rad) with TaqMan Gene Expression Assay (Applied Biosystems) for *HPRT*, *CCL11*, *CCL24*, and *CCL26*. Data were analyzed by normalizing threshold cycles to HPRT.

### **ELISA**

Cell culture supernatants were assayed for CCL11 using the Mouse CCL11/Eotaxin Quantikine ELISA Kit (R&D systems) and for CCL24 using the Eotaxin-2 (CCL24) Mouse ELISA Kit (Abcam) according to manufacturer's instructions.

### **Histology and immunohistochemistry**

Mouse hearts were cut in half, fixed in SafeFix (Thermo Fisher Scientific), paraffin embedded, and cut into 5- $\mu$ m-thick sections (HistoServ). Serial sections were stained with 5  $\mu$ g/ml polyclonal goat anti-mouse CCL11 or CCL24 antibodies (R&D Systems) using the Anti-Goat HRP-DAB Cell & Tissue Staining Kit (R&D Systems) according to manufacturer's protocol and counterstained with Hematoxylin. The number of infiltrating eosinophils was determined by counting eosinophils on 40X images of H&E-stained biopsy sections. The number of eosinophils was averaged over the area observed.

### **Statistics**

Two groups were analyzed using Student's *t*-test for normally distributed data or Mann-Whitney test for nonparametric data. Multiple group analysis was performed by one-way ANOVA followed by Tukey's multiple comparisons test or Dunnett's multiple comparisons test. Calculations were performed in Prism 6

(GraphPad Software Inc.). p values <0.05 were considered statistically significant and are denoted by asterisks: \*p<0.05, \*\*p<0.01, \*\*\*p<0.001.

## Results

### Eosinophils localize to the heart in response to eotaxin-CCR3 signaling

We hypothesized that the eotaxin-CCR3 pathway is important for eosinophil trafficking to the heart based on our previous observations that *Ccl11* (eotaxin-1) and *Ccl24* (eotaxin-2) expression are substantially increased in IFN $\gamma$ <sup>-/-</sup>IL-17A<sup>-/-</sup> mice with eosinophilic myocarditis compared to WT mice during EAM<sup>189</sup>. By including additional groups, we determined that this expression pattern also occurs in the absence of eosinophils in  $\Delta$ dblGATA1 and IFN $\gamma$ <sup>-/-</sup>IL-17A<sup>-/-</sup> $\Delta$ dblGATA1 mice (Figure 6A). IFN $\gamma$ <sup>-/-</sup>IL-17A<sup>-/-</sup> $\Delta$ dblGATA1 mice showed substantially higher cardiac expression of *Ccl11* and *Ccl24* than  $\Delta$ dblGATA1 mice at day 21 of EAM. This finding establishes that eosinophils are not required for eotaxin expression in the heart during EAM.

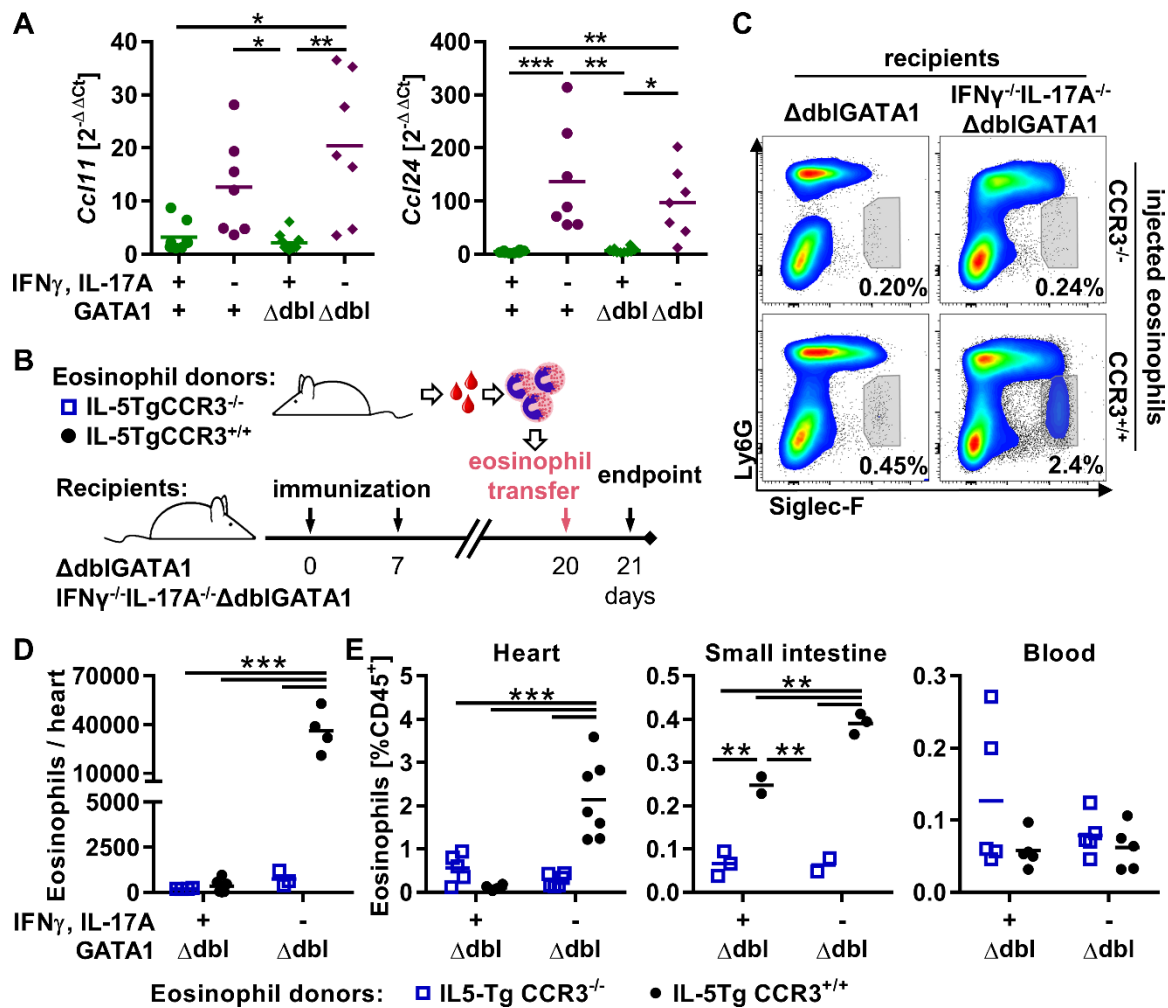
We employed adoptive cell transfer to determine the importance of the eotaxin receptor CCR3 in eosinophil trafficking to the heart (Figure 6B). Eosinophils were isolated from IL-5 transgenic mice (IL-5Tg) because these mice develop massive eosinophilia<sup>195</sup>. Recipient  $\Delta$ dblGATA1 and IFN $\gamma$ <sup>-/-</sup>IL-17A<sup>-/-</sup> $\Delta$ dblGATA1 mice deficient in eosinophils were immunized on days 0 and 7 to induce EAM. On day 20, eosinophils were isolated from peripheral blood of IL-5TgCCR3<sup>+/+</sup> and IL-5TgCCR3<sup>-/-</sup> donor mice and 10<sup>7</sup> donor cells were injected intravenously into recipients. The following day, recipients were sacrificed. Eosinophil infiltration in multiple organs was quantified by flow cytometry (Figure 6C-E, Figure 7A-B). All eosinophils recovered in the recipients were necessarily donor-derived because the  $\Delta$ dblGATA1 mutation blocks eosinophil development in the bone marrow<sup>194</sup>. Only when CCR3-expressing eosinophils were injected into IFN $\gamma$ <sup>-/-</sup>IL-17A<sup>-/-</sup> $\Delta$ dblGATA1 recipients, could significant numbers of eosinophils be retrieved from the heart (Figure 6C, D). This combination of donor and recipient alone resulted in a significant increase in cardiac eosinophils in both frequency (Figure 6C, E) and absolute numbers (Figure 6D). Use of bone marrow-derived eosinophils

from WT and CCR3<sup>-/-</sup> donors for adoptive transfer yielded the same results (*data not shown*). Expression of CCR3 by eosinophils allowed for substantial eosinophil trafficking to the heart, but only in the presence of high cardiac eotaxin expression. Therefore, two conditions are required for efficient eosinophil trafficking to the heart: substantial eotaxin expression in the heart as in the IFN $\gamma$ <sup>-/-</sup>IL-17A<sup>-/-</sup> $\Delta$ dblGATA1 mice and expression of the CCR3 receptor on eosinophils. These data led us to conclude that the CCR3-eotaxin pathway is essential for eosinophil trafficking to the heart.

### **Eosinophils reach highest frequency in the hearts of IFN $\gamma$ <sup>-/-</sup>IL-17A<sup>-/-</sup> $\Delta$ dblGATA1 mice**

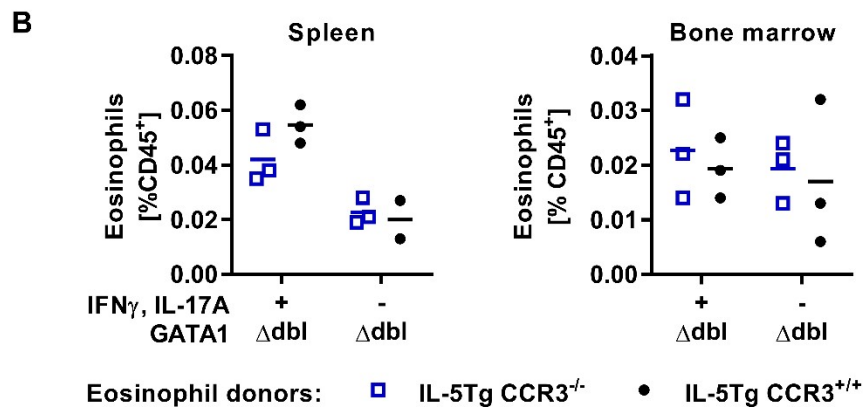
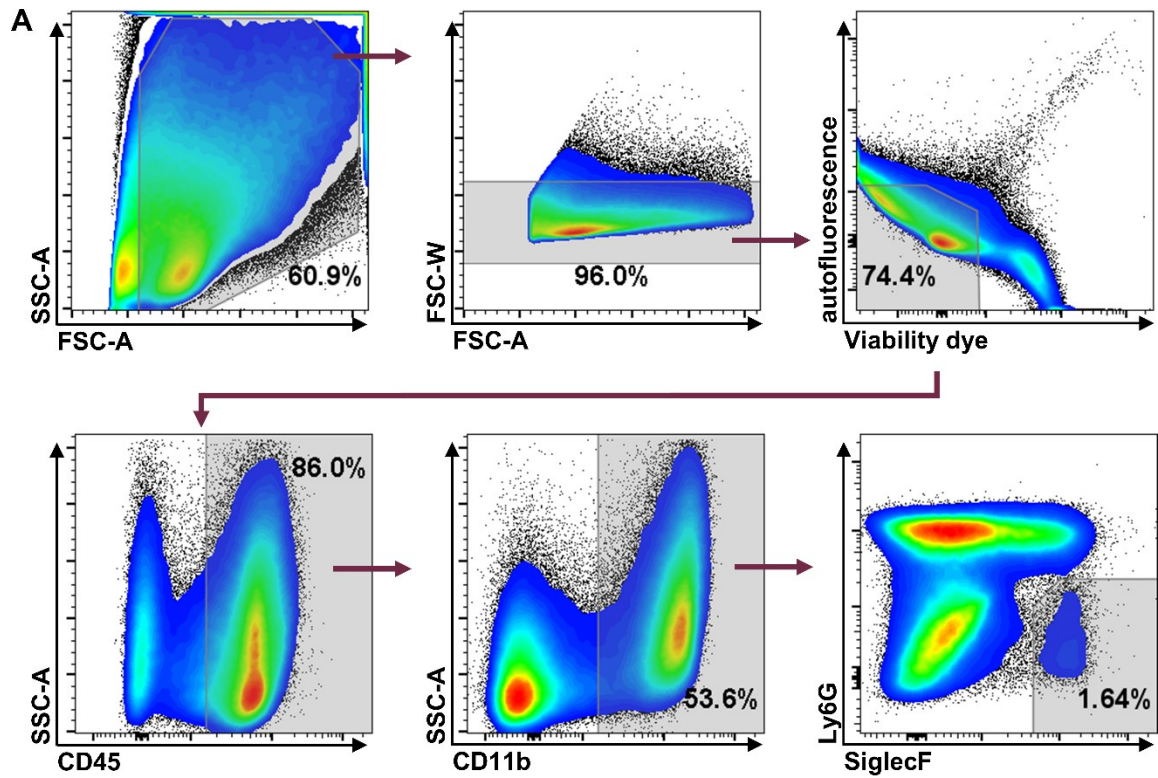
To determine whether eosinophil migration into tissues in response to the eotaxin-CCR3 pathway was intact in  $\Delta$ dblGATA1 recipients, we analyzed eosinophil numbers in the small intestine in parallel with the heart. The intestine harbors eosinophils under steady-state conditions in mice and humans<sup>192, 199</sup>. Eosinophil trafficking to the intestine is known to be dependent on the eotaxin-CCR3 pathway<sup>69</sup>. CCR3-expressing eosinophils were found at significantly higher frequencies in the intestines of both recipients compared to CCR3-deficient eosinophils (Figure 1E). Adoptively transferred eosinophils were also retrieved from peripheral blood, spleen, and bone marrow at low frequencies (Figure 6E, Figure 8, Figure 7B). A role for the eotaxin-CCR3 pathway has not been described for these organs, and we did not find more CCR3<sup>+/+</sup> eosinophils compared to CCR3<sup>-/-</sup> eosinophils in any of these tissues (Figure 6E, Figure 7B).

Importantly, transferred CCR3<sup>+/+</sup> eosinophils in IFN $\gamma$ <sup>-/-</sup>IL-17A<sup>-/-</sup> $\Delta$ dblGATA1 recipients reached higher frequencies in the heart than any other organ (Figure 8A). Eosinophil frequency in the heart was about 10- to 20-fold higher than in the blood or spleen and about 2-fold higher than in the intestine. In contrast, in  $\Delta$ dblGATA1 recipients transferred CCR3<sup>+/+</sup> eosinophils were increased in the small intestine compared to all other organs, including the heart (Figure 8B). Thus, eosinophils are specifically recruited to the heart by the Th2-driven myocarditis that develops in the absence of IFN $\gamma$  and IL-17A.



**Figure 6: CCR3 is required for eosinophil trafficking to the heart in eosinophilic myocarditis**

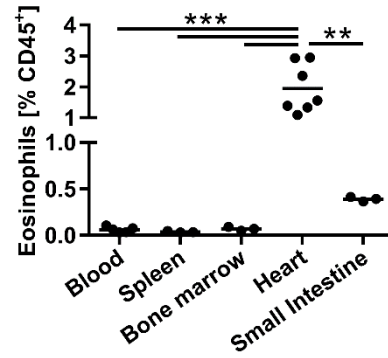
A) Expression of indicated genes was analyzed by qPCR from heart homogenates at day 21 post-immunization and is expressed as  $2^{-\Delta\Delta Ct}$  values relative to *Gapdh* and naïve WT mice. B) Schematic of adoptive eosinophil transfer into immunized recipient mice for data depicted in (C-D). C) Representative bivariate flow cytometry plots showing SiglecF<sup>+</sup>Ly6G<sup>lo</sup> eosinophils as a proportion of viable CD45<sup>+</sup> heart-infiltrating cells following transfer. D) Total number of heart-infiltrating eosinophils after eosinophil transfer. E) Frequency of eosinophils in different organs following adoptive transfer. Groups were compared by one-way ANOVA followed by Tukey's multiple comparison. Data points represent individual animals, bars indicate means. Data are representative of 2-4 independent experiments. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .



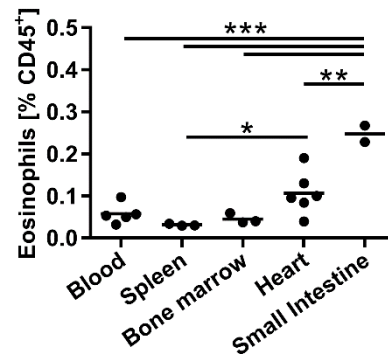
**Figure 7: Gating strategy of eosinophils**

Eosinophil frequency in indicated organs one day following adoptive transfer of CCR3<sup>+/+</sup> eosinophils into IFN $\gamma$ <sup>-/-</sup>IL-17A<sup>-/-</sup> $\Delta$ dblGATA1 recipients (A) and  $\Delta$ dblGATA1 recipients (B). The same experiment as in Figure 1E is shown. Groups were compared by one-way ANOVA followed by Tukey's multiple comparison. Data points represent individual animals, bars indicate means. Data are representative of 2 independent experiments. \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001.

**A**  $\text{IFN}\gamma^{-/-}\text{IL-17A}^{-/-}\Delta\text{dbIGATA1}$  recipients



**B**  $\Delta\text{dbIGATA1}$  recipients



**Figure 8: Adoptively transferred eosinophils reach highest frequency in the heart**

Eosinophil frequency in indicated organs one day following adoptive transfer of  $\text{CCR3}^{+/+}$  eosinophils into  $\text{IFN}\gamma^{-/-}\text{IL-17A}^{-/-}\Delta\text{dbIGATA1}$  recipients (A) and  $\Delta\text{dbIGATA1}$  recipients (B). The same experiment as in Figure 6E is shown. Groups were compared by one-way ANOVA followed by Tukey's multiple comparison. Data points represent individual animals, bars indicate means. Data are representative of 2 independent experiments. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .

### **Genetic ablation of CCR3 in IFN $\gamma$ <sup>-/-</sup>IL-17A<sup>-/-</sup> mice blocks eosinophil trafficking to the heart**

To further test our hypothesis that eosinophil trafficking to the heart is mainly CCR3-dependent in eosinophilic myocarditis, we crossed IFN $\gamma$ <sup>-/-</sup>IL-17A<sup>-/-</sup> mice with CCR3<sup>-/-</sup> mice. Eosinophil frequencies in the heart were dramatically reduced from 24% in IFN $\gamma$ <sup>-/-</sup>IL-17A<sup>-/-</sup> mice to 2% in IFN $\gamma$ <sup>-/-</sup>IL-17A<sup>-/-</sup>CCR3<sup>-/-</sup> mice on day 21 of EAM (Figure 9A, B). Eosinophil frequencies in the spleens did not differ between the two strains (Figure 9C). Although considerably diminished, eosinophils were not entirely absent from the hearts of IFN $\gamma$ <sup>-/-</sup>IL-17A<sup>-/-</sup>CCR3<sup>-/-</sup> mice (Figure 9A, B) and accounted for similar frequencies in heart and spleen on day 21 of EAM (Figure 9B, C). This suggests that there is no specific recruitment of eosinophils to the heart in the absence of CCR3.

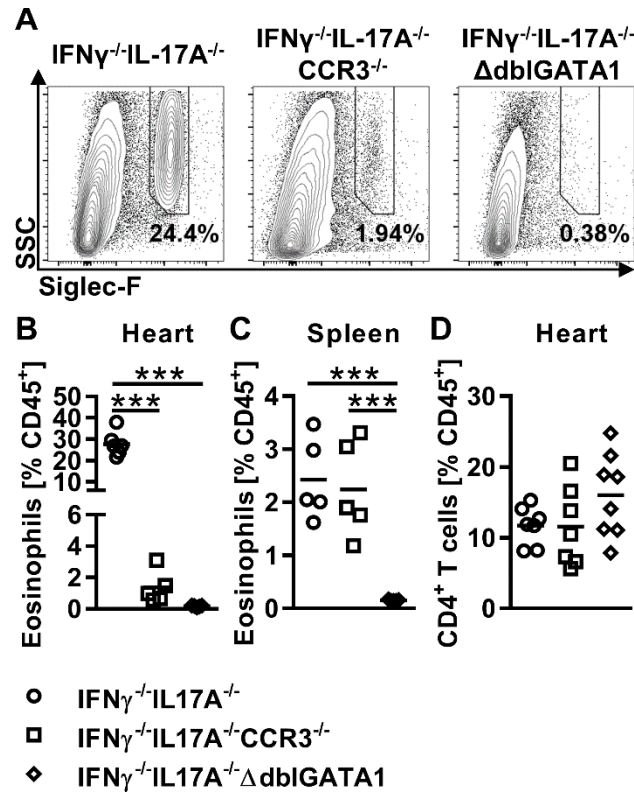
Expression of CCR3 has also been reported on human Th2 cells<sup>79</sup>. We observed similar CD4<sup>+</sup> T cell frequencies in the presence or absence of CCR3 during EAM (Figure 9D). This indicates that reduced eosinophil trafficking to the heart in IFN $\gamma$ <sup>-/-</sup>IL-17A<sup>-/-</sup>CCR3<sup>-/-</sup> mice is not a consequence of decreased T cell infiltration. Moreover, all 3 strains developed myocarditis of similar histopathologic severity (*data not shown*). Taken together, these findings demonstrate that in eosinophilic myocarditis without peripheral eosinophilia, as is observed in IFN $\gamma$ <sup>-/-</sup>IL-17A<sup>-/-</sup> mice, eosinophil trafficking to the heart is dependent on the eotaxin-CCR3 pathway.

### **CCL11 and CCL24 are expressed by different cell types in the heart**

Immunohistochemical staining for eotaxins on serial heart sections revealed interstitial expression of CCL11 at day 21 of EAM in IFN $\gamma$ <sup>-/-</sup>IL-17A<sup>-/-</sup> $\Delta$ dbpGATA1 mice (Figure 10A),  $\Delta$ dbpGATA1 mice (Figure 10E), and naïve mice of both strains (*data not shown*). Only single CCL11<sup>+</sup> cells were observed in inflammatory foci (Figure 10B, F). This pattern is consistent with expression by fibroblasts. CCL24, in contrast, was not detected interstitially between cardiomyocytes (Figure 10C, G). Instead, CCL24 localized to areas of inflammation in IFN $\gamma$ <sup>-/-</sup>IL-17A<sup>-/-</sup> $\Delta$ dbpGATA1 mice (Figure 10D), consistent with expression by heart infiltrating cells. CCL24 was not detectable in  $\Delta$ dbpGATA1 mice (Figure 10G, H) or naïve mouse hearts of either genotype (*data not*



shown). This demonstrates that CCL11 and CCL24 are expressed by different cell types in the heart and under different conditions.



**Figure 9: Genetic ablation of CCR3 in IFN $\gamma$ <sup>-/-</sup>IL-17A<sup>-/-</sup> mice reduces eosinophil frequency in the heart**

A) Representative bivariate flow cytometry plots of heart-infiltrating CD45<sup>+</sup> cells. Gates show frequency of SiglecF<sup>+</sup>SSC<sup>hi</sup> eosinophils. B-D) Eosinophil and T helper cell frequency in heart and spleen at day 21 post-immunization in the indicated strains. Groups were compared by one-way ANOVA followed by Tukey's multiple comparison. Data points represent individual animals, bars indicate means. Data are representative of 2 independent experiments. \*\*\*p < 0.001.

### **Cardiac fibroblasts are major CCL11 producers in the heart**

Next, we sought to establish which cell types specifically were responsible for eotaxin production. We used a *Ccl11* mRNA probe for flow cytometry to address this question in an unbiased manner. We included surface markers for the identification of tissue resident cells and infiltrating hematopoietic-lineage cells (Figure 11). Out of the different cell populations in the heart at day 21 of EAM, the highest expression of *Ccl11* was detected in CD45<sup>+</sup>CD31<sup>+</sup>CD140a<sup>+</sup> cardiac fibroblasts (Figure 10I, J). This fibroblast subset showed significantly higher *Ccl11* expression compared to total viable cells for both IFN $\gamma$ <sup>-/-</sup>IL-17A<sup>-/-</sup> $\Delta$ dblGATA1 and  $\Delta$ dblGATA1 mice (Figure 10J). Low levels of *Ccl11* expression could be detected by qPCR even in naïve hearts. The highest *Ccl11* signal in naïve heart cells was also in the CD140a<sup>+</sup> fibroblast subset (*data not shown*). Thus, cardiac fibroblasts are the major producer of *Ccl11* in the heart during steady-state and in myocarditis. To confirm these results with an independent method, we sorted several cell populations from  $\Delta$ dblGATA1 and IFN $\gamma$ <sup>-/-</sup>IL-17A<sup>-/-</sup> $\Delta$ dblGATA1 mice at day 21 of EAM and analyzed eotaxin expression by qPCR. CD31<sup>+</sup> endothelial cells were depleted before cells were sorted by FACS (Figure 12A). Again, CD45<sup>+</sup>CD140a<sup>+</sup> fibroblasts showed the highest expression of *Ccl11* compared to all other sorted cells (Figure 10K). In conclusion, the major producers of CCL11 are CD140a<sup>+</sup> cardiac fibroblasts located interstitially between myocytes. These CCL11-expressing fibroblasts were found in both  $\Delta$ dblGATA1 and IFN $\gamma$ <sup>-/-</sup>IL-17A<sup>-/-</sup> $\Delta$ dblGATA1 mice at steady-state and in EAM.

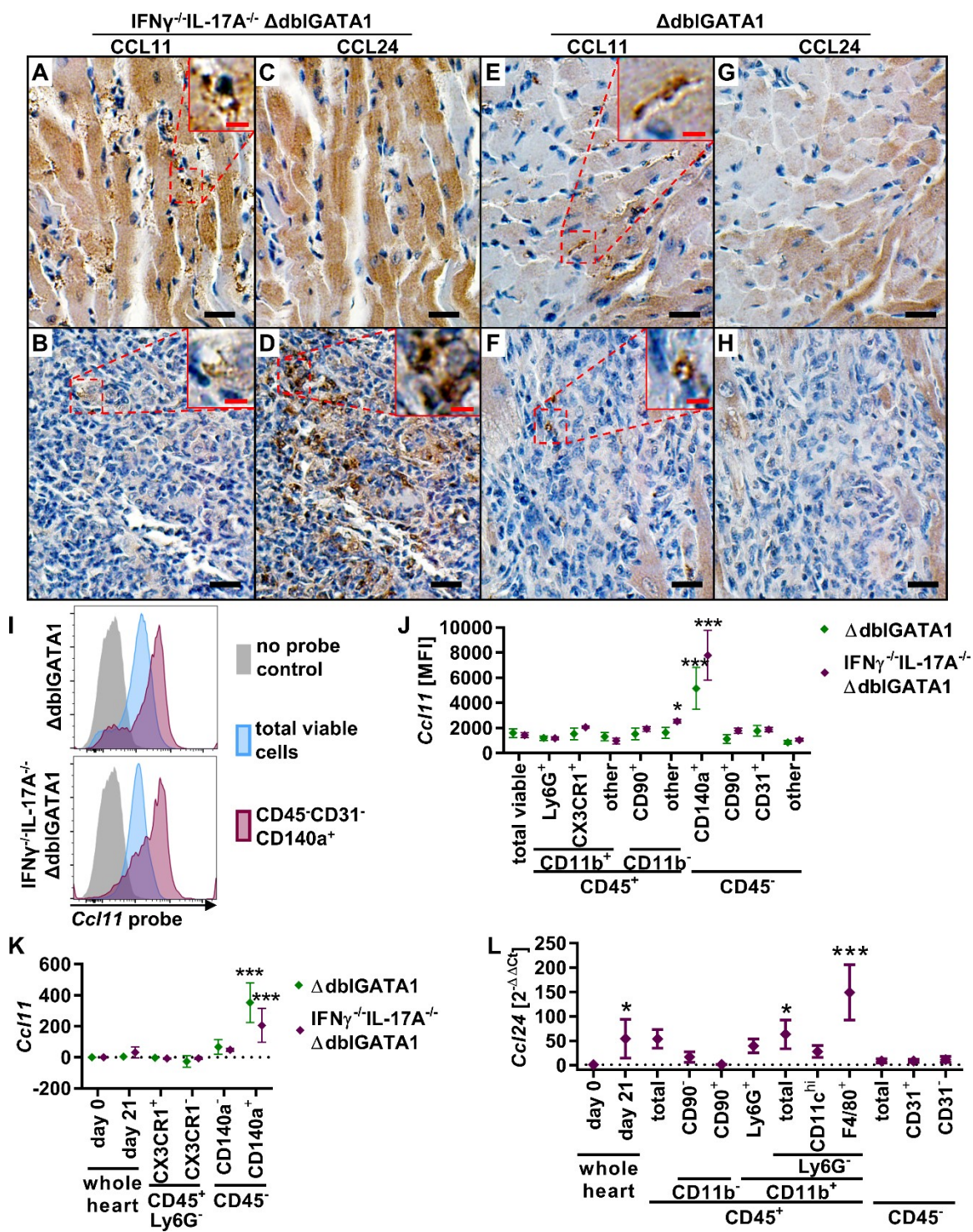
### **CCL24 is produced by F4/80<sup>+</sup> macrophages during myocarditis**

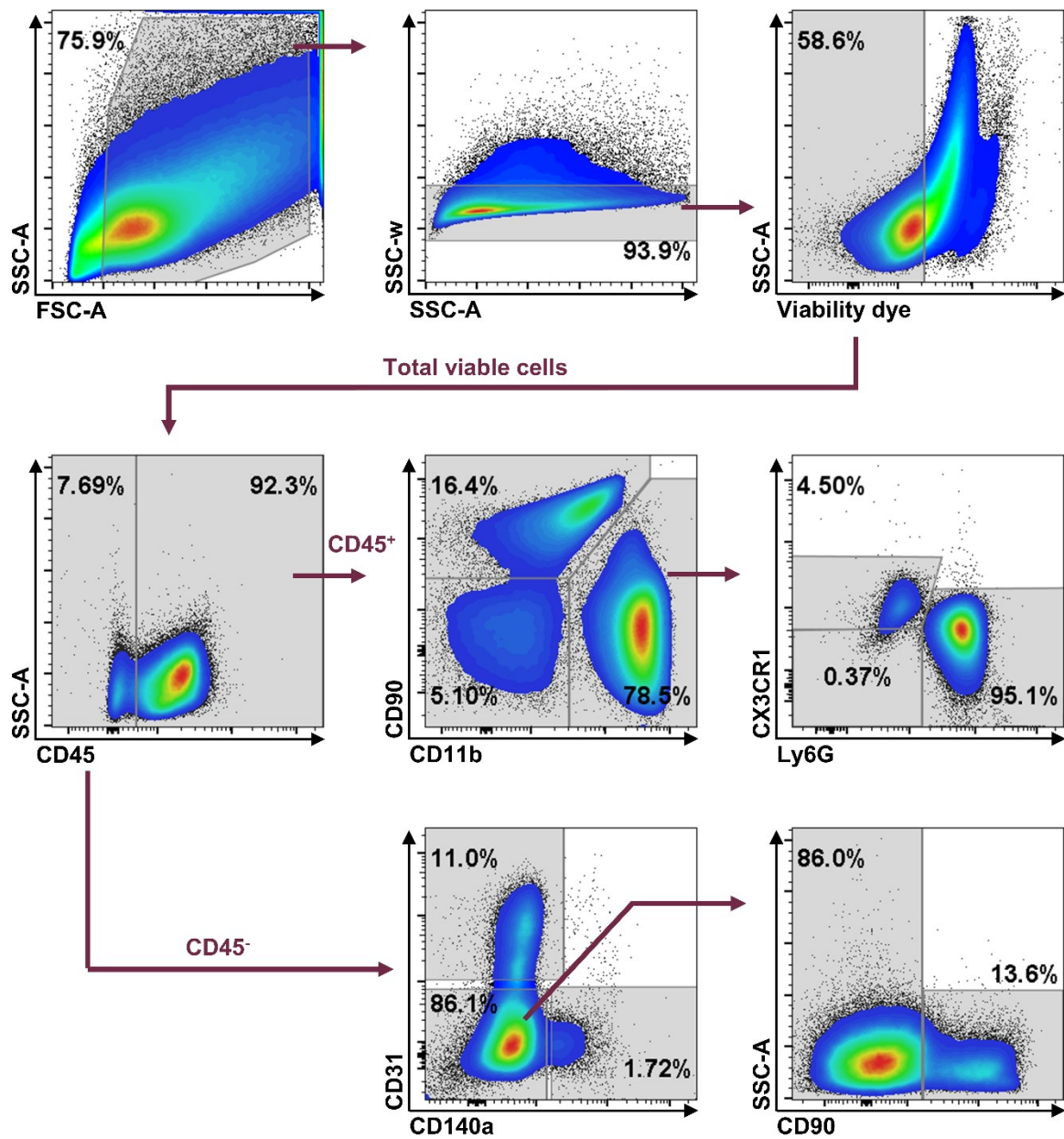
Unlike *Ccl11*, which was upregulated 4-fold during EAM in  $\Delta$ dblGATA1 compared to naïve mice, *Ccl24* did not increase in EAM in  $\Delta$ dblGATA1 mice (Figure 12B). In the IFN $\gamma$ <sup>-/-</sup>IL-17A<sup>-/-</sup> $\Delta$ dblGATA1 mice, however, *Ccl24* was highly upregulated during EAM (Figure 10L, Figure 12B). The populations shown in Figure 10K did not conclusively reveal the major *Ccl24* expressing cell type (Figure 12B). Therefore, we sorted 10 overlapping populations (Figure 13) and assayed them for *Ccl24* expression. CD45<sup>+</sup>CD11b<sup>+</sup>Ly6G<sup>+</sup>F4/80<sup>+</sup> macrophages were the major *Ccl24* expressing cells in EAM in IFN $\gamma$ <sup>-/-</sup>IL-17A<sup>-/-</sup> $\Delta$ dblGATA1 mice (Figure 10L). Expression of

CCL24 by macrophages is consistent with localization to inflammatory foci observed by immunohistochemistry (Figure 10). The staining for eotaxin proteins on serial sections (Figure 10A-H) and qPCR from different sorted cell populations (Figure 10K, L, Figure 14) clearly demonstrated that CCL11 and CCL24 were expressed by different cell types during EAM. CCL24 was produced by F4/80<sup>+</sup> macrophages, localized in foci of inflammation, and detected as protein only in immunized IFN $\gamma$ <sup>-/-</sup>IL-17A<sup>-/-</sup> $\Delta$ dblGATA1 mice.

**Figure 10: CCL11 is expressed by cardiac fibroblasts while CCL24 is expressed by F4/80<sup>+</sup> macrophages during eosinophilic myocarditis**

A-H) Immunohistochemistry staining for CCL11 (A-B, E-F) and CCL24 (C-D, G-H) of heart sections from day 21 in IFN $\gamma$ <sup>-/-</sup>IL-17A<sup>-/-</sup> $\Delta$ dblGATA1 mice (A-D) and  $\Delta$ dblGATA1 mice (E-H) showing areas without (A, C, E, G) and with inflammation (B, D, F, H). Pictures are representative of 2-3 mice per group. Black scale bars: 20  $\mu$ m, red scale bars: 5  $\mu$ m. I) Ccl11 expression was detected using a probe against Ccl11 mRNA in the FlowRNA II Assay and is shown for total viable cells (blue) and CD140a<sup>+</sup> fibroblasts (red) at day 21 post-immunization from concatenated samples from 5-6 mice per group. J) Mean fluorescence intensity (MFI) for Ccl11 probe in total viable cells and indicated cell populations. Mean  $\pm$  SD of 5-6 mice per group. K) Ccl11 expression in cells sorted from 3 individual mice per group as in Supplemental Figure 3. L) Ccl24 expression in cells sorted from 4 individual mice as in Supplemental Figure 4. K, L) Eotaxin expression was analyzed by qPCR and normalized to Gapdh and naïve mouse heart homogenates, shown is mean  $\pm$  SD. Gene expression in heart homogenates from naïve and immunized mice are included for comparison. J-L) Groups were compared by one-way ANOVA followed by Dunnett's multiple comparison test comparing all subsets to total viable cells (J) or to naïve heart (K, L) for each genotype. \*p < 0.05, \*\*\*p < 0.001.

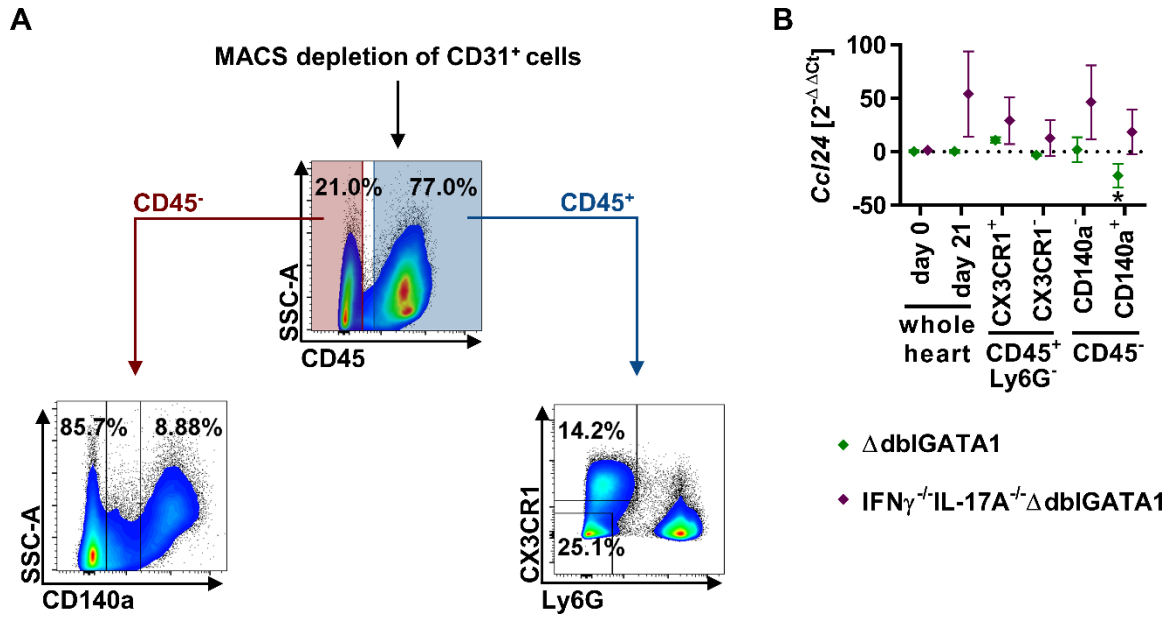




**Figure 11: Gating strategy for prime flow RNA assay**

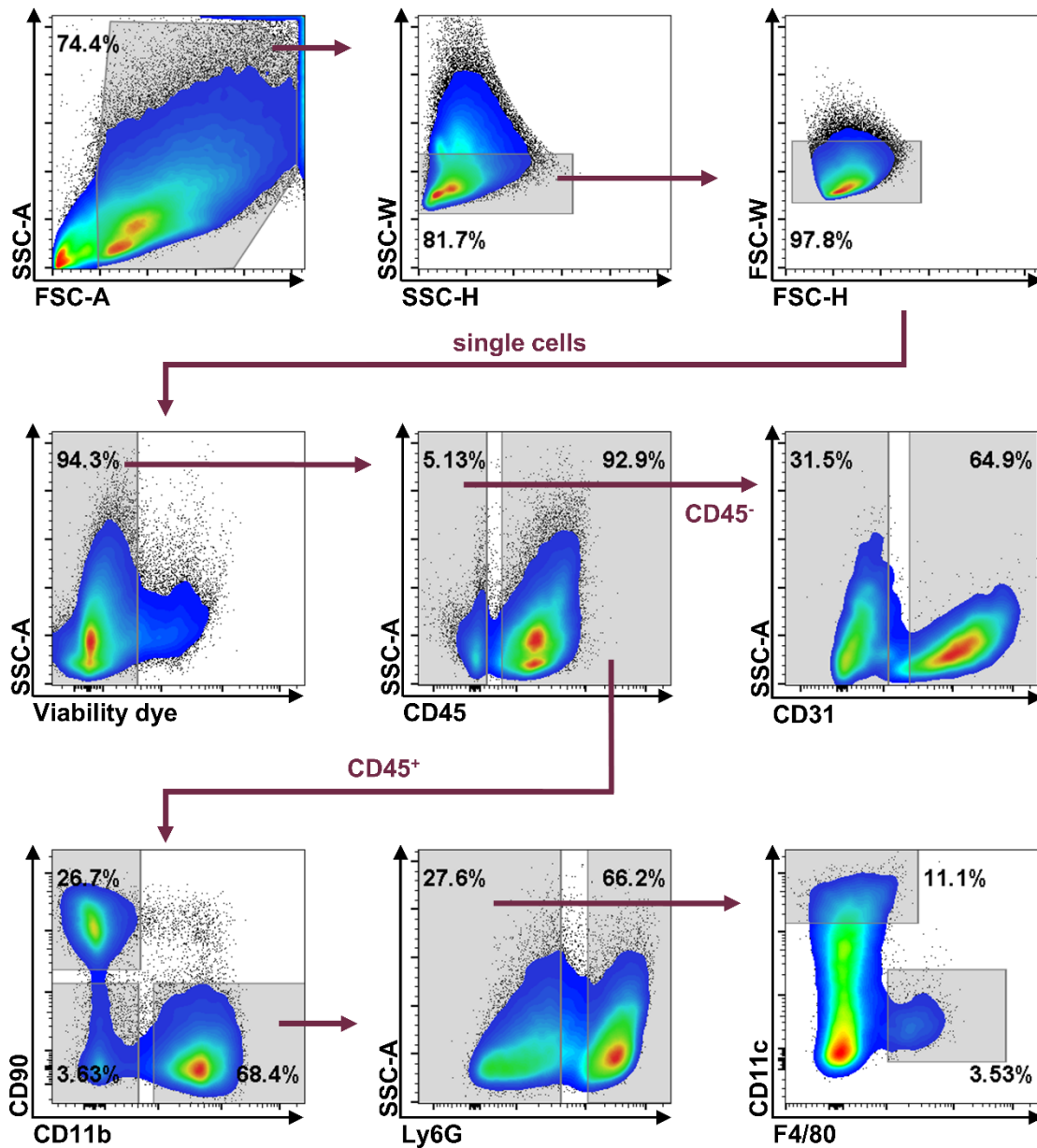
Gating strategy is shown representatively for an IFN $\gamma$ <sup>-/-</sup>IL-17A<sup>-/-</sup> $\Delta$ blGATA1 mouse. Mean fluorescence intensity for the *Cd11* probe in Figure 4J was determined for each cell population starting with total viable cells.





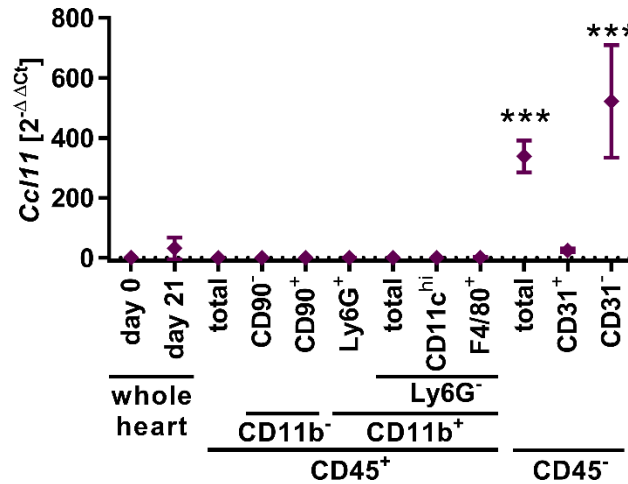
**Figure 12: Flow sorting of cardiac resident and cardiac infiltrating cells**

A) Gating strategy is shown representatively for an IFN $\gamma$ <sup>-/-</sup>IL-17A<sup>-/-</sup>ΔdbIGATA1 mouse. Cells were isolated from hearts at day 21 of EAM, first depleted of endothelial cells using anti-CD31 beads and then sorted into 4 populations: CD45<sup>-</sup>CD140a<sup>-</sup>, CD45<sup>-</sup>CD140a<sup>+</sup>, CD45<sup>+</sup>CX3CR1<sup>+</sup>Ly6G<sup>-</sup>, CD45<sup>+</sup>CX3CR1<sup>-</sup>Ly6G<sup>-</sup>. B), *Ccl24* expression in sorted cells and whole heart homogenates was analyzed by qPCR and normalized to *Gapdh* and naïve heart homogenates. Cells were sorted from 3 individual mice, shown is mean  $\pm$  SD. Groups were compared by one-way ANOVA followed by Dunnett's multiple comparison comparing all subsets to naïve heart for each genotype.



**Figure 13: Gating strategy for flow sorting of heart infiltrating and resident cell populations**

Gating strategy is shown representatively for an  $\text{IFN}\gamma^{-/-}\text{IL-17A}^{-/-}\Delta\text{dblGATA1}$  mouse. A total of 10 overlapping populations were sorted:  $\text{CD45}^{-}$ ,  $\text{CD45}^{-}\text{CD31}^{-}$ ,  $\text{CD45}^{-}\text{CD31}^{+}$ ,  $\text{CD45}^{+}$ ,  $\text{CD45}^{+}\text{CD90}^{+}\text{CD11b}^{-}$ ,  $\text{CD45}^{+}\text{CD90}^{-}\text{CD11b}^{-}$ ,  $\text{CD45}^{+}\text{CD90}^{-}\text{CD11b}^{+}$ ,  $\text{CD45}^{+}\text{CD90}^{-}\text{CD11b}^{+}\text{Ly6G}^{+}$ ,  $\text{CD45}^{+}\text{CD90}^{-}\text{CD11b}^{+}\text{Ly6G}^{-}\text{CD11c}^{\text{hi}}$ ,  $\text{CD45}^{+}\text{CD90}^{-}\text{CD11b}^{+}\text{Ly6G}^{-}\text{F4/80}^{+}$ .



**Figure 14: *Ccl11* expression in sorted cells**

Cells were sorted from IFN $\gamma$ <sup>-/-</sup>IL-17A<sup>-/-</sup> $\Delta$ dblGATA1 mice (n=4) as depicted in Supplemental Figure 4, shown is mean  $\pm$  SD. *Ccl11* expression in sorted cells and whole heart homogenates was analyzed by qPCR and normalized to *Gapdh* and naïve heart homogenates. Groups were compared by one-way ANOVA followed by Dunnett's multiple comparison comparing all subsets to naïve heart. \*\*\*p < 0.001.

#### **Primary cardiac fibroblasts produce eotaxins *in vitro* in response to IL-4 and IL-13**

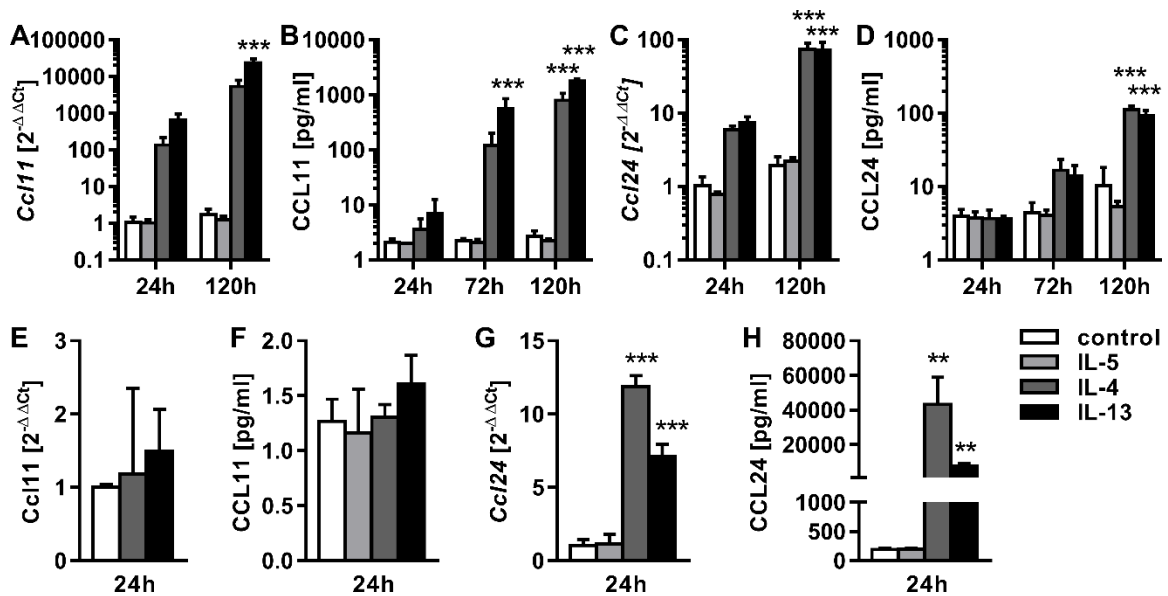
We isolated primary fibroblasts from the hearts of naïve adult WT mice to determine if cardiac fibroblasts could produce CCL11 in response to Th2 cytokines. *In vitro* culture of fibroblasts with the Th2 cytokines IL-4 and IL-13, but not IL-5, induced *Ccl11* mRNA expression (Figure 15A) and secretion into the cell culture medium (Figure 15B). At 100 ng/ml, IL-13 was more potent than IL-4 in inducing CCL11 at the late time points (Figure 15A, B). The Th1 and Th17 cytokines IFN $\gamma$  and IL-17A did not induce CCL11 (*data not shown*). This confirms that cardiac fibroblasts produce CCL11 and do so in response to key Th2 cytokines IL-4 and IL-13.

Surprisingly, IL-4 and IL-13 also induced CCL24 expression in cardiac fibroblasts (Figure 15C, D) although at a much lower magnitude than CCL11. This is consistent with the observation that higher expression of CCL24 was detected in CD140a<sup>+</sup> fibroblasts of IFN $\gamma$ <sup>-/-</sup>IL-17A<sup>-/-</sup> $\Delta$ dblGATA1 mice compared to  $\Delta$ dblGATA1 mice (Figure 12B).



### IL-4 and IL-13 induce CCL24 in macrophages

To determine if Th2 cytokines could also induce eotaxins in macrophages, we cultured bone marrow-derived macrophages with different cytokines. As expected, IFN $\gamma$  and IL-17A did not induce eotaxins in macrophages (*data not shown*). CCL11 was not induced in Macrophages in response to Th2 cytokines (Figure 15E, F). However, they secreted large amounts of CCL24 after stimulation with IL-4 and IL-13 (Figure 15G, H). These data support macrophages as potent producers of CCL24. The increased expression of IL-4 and IL-13 from T cells in IFN $\gamma$ <sup>-/-</sup>IL-17A<sup>-/-</sup> compared to WT mice<sup>189</sup> may explain the increased eotaxin expression in these mice.

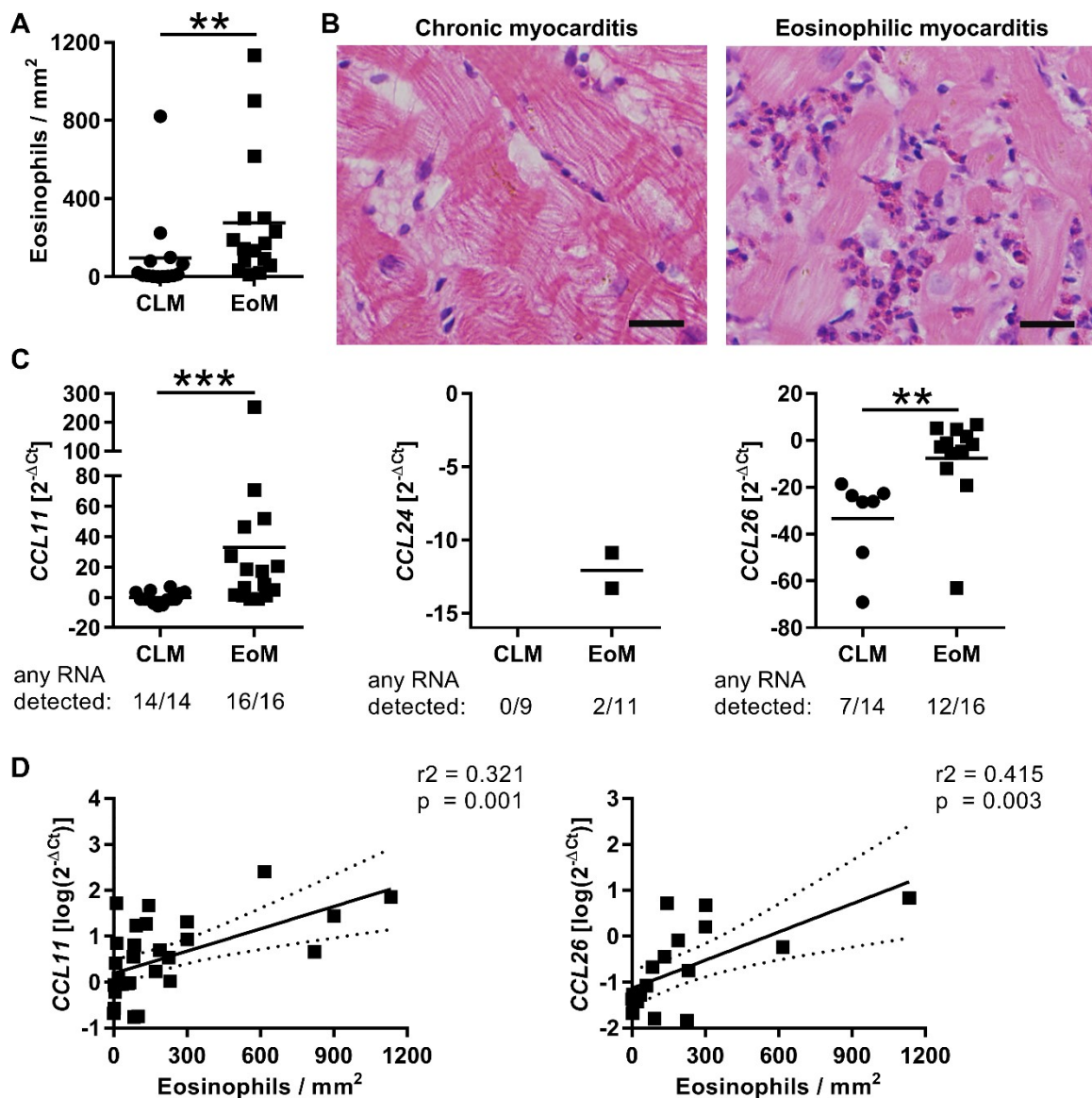


**Figure 15: IL-4 and IL-13 induce CCL11 in cardiac fibroblasts and CCL24 in macrophages**

Adult mouse cardiac fibroblasts (A-D) and bone marrow-derived macrophages (E-H) from naïve WT mice were cultured in the presence of indicated cytokines at 100 ng/ml or without cytokines (control). (A, C, E, G) *Ccl11* and *Ccl24* mRNA expression was measured by qPCR and normalized to *Gapdh* and untreated controls. (B, D, F, H) Eotaxin concentrations in cell culture supernatants were measured by ELISA at indicated time points. Groups were compared by one-way ANOVA followed by Dunnett's multiple comparison. All groups were compared to untreated controls at day 1. Shown is mean  $\pm$  SD. Data are representative of 2-3 independent experiments. \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .

### **Cardiac eotaxin expression is increased in patients with eosinophilic myocarditis**

To test whether eotaxins are also important for eosinophil trafficking in patients with eosinophilic myocarditis, we analyzed endomyocardial biopsy samples. Patients with eosinophilic myocarditis had increased numbers of heart-infiltrating eosinophils compared to patients with chronic lymphocytic myocarditis (Figure 16A, B). Expression of *CCL11* and *CCL26* (an eotaxin specific to humans and a pseudogene in mice) was increased in eosinophilic myocarditis patients compared to chronic lymphocytic myocarditis patients (Figure 16C). *CCL11* was detected in all samples regardless of diagnosis. In contrast, *CCL26* was detected in 75% of eosinophilic myocarditis patients but only in 50% of chronic lymphocytic myocarditis patients (Figure 16C). *CCL24* was detected in some of the eosinophilic myocarditis biopsies but in none of the chronic lymphocytic myocarditis samples (Figure 16C). It is possible that *CCL24* is expressed in the heart only in the context of eosinophilic myocarditis. Expression of *CCL11* and *CCL26* correlated positively with the number of infiltrating eosinophils in the biopsies (Figure 16D). The significant increase in eotaxin expression in the hearts of patients with eosinophilic myocarditis and the correlation of eotaxin expression with eosinophil infiltration suggest that eotaxins are important for eosinophil trafficking to the human heart during eosinophilic myocarditis.

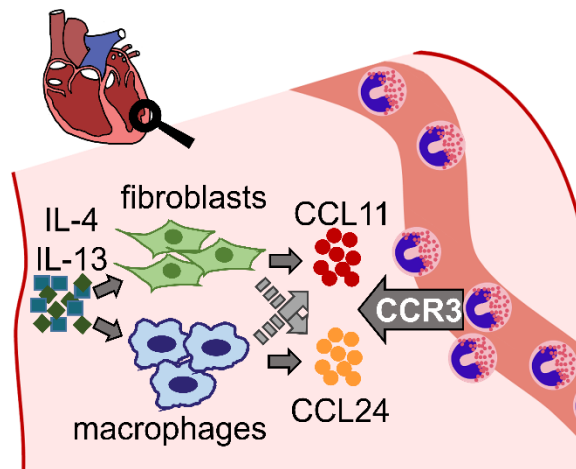


**Figure 16: Eotaxins are increased in the hearts of patients with eosinophilic myocarditis**

A) Eosinophil infiltration was quantified on H&E-stained biopsy sections. B) Representative images of (A). C) Eotaxin mRNA expression in endomyocardial biopsy samples is shown as fold expression relative to HPRT (2<sup>-ΔCt</sup>). A, C) Groups were compared by Mann-Whitney *U*-test. D) Linear regression of eotaxin mRNA expression on the number of infiltrating eosinophils. Dashed lines indicate 95% confidence intervals. CLM, chronic lymphocytic myocarditis; EoM, eosinophilic myocarditis. Scale bars: 20 μm. \*\**p* < 0.01, \*\*\**p* < 0.001.

## Discussion

Eosinophils can infiltrate many different organs in response to multiple local stimuli, such as chemokines and lipid mediators<sup>190</sup>. In this study, we present evidence that eosinophil trafficking to the heart in eosinophilic myocarditis depends on the chemokine receptor CCR3 and expression of its ligands CCL11, CCL24, and CCL26 in the heart in a mouse model and in patients with eosinophilic myocarditis. This is the first description of a precise pathway that recruits eosinophils to the heart. We identified the cell types producing eotaxins in the heart and showed that their expression in cardiac fibroblasts and macrophages is controlled by two cytokines, IL-4 and IL-13 (Figure 17). These findings enhance our understanding of how eosinophils contribute to cardiac pathology and provide insights into the regulation of eosinophilic heart disease and eosinophilic autoimmune diseases in general. Moreover, it opens new therapeutic avenues to prevent eosinophil-mediated heart damage.



**Figure 17: Model of eosinophil trafficking to the heart during eosinophilic myocarditis**

We demonstrated that CCR3 is required for eosinophil trafficking to the heart using adoptive transfer and genetic methods.  $\text{IFN}\gamma^{-/-}\text{IL-17A}^{-/-}\text{CCR3}^{-/-}$  mice showed a dramatic reduction of eosinophils in the heart compared to  $\text{IFN}\gamma^{-/-}\text{IL-17A}^{-/-}$  mice. In addition, only  $\text{CCR3}^{+/+}$ , but not  $\text{CCR3}^{-/-}$ , adoptively transferred

eosinophils were recovered from the heart of eosinophil-deficient recipients with myocarditis. Using an adoptive transfer system permitted us to exclude effects of local eosinophil proliferation and positive feedback loops, such as eosinophil-derived IL-4 and IL-13 increasing local eotaxin production by other cell types. Additionally, this approach allowed us to distinguish between consequences of CCR3 expression in eosinophils versus other cell types, which is still a controversial issue in the mouse<sup>200, 201</sup>.

While CCR3 in humans can bind ligands other than eotaxins, mouse CCR3 is only known to bind the mouse chemokines CCL11 and CCL24<sup>190, 191</sup>. Together with the results from our adoptive transfer experiments, this implies that the high eotaxin expression in IFN $\gamma$ <sup>-/-</sup>IL-17A<sup>-/-</sup> $\Delta$ dblGATA1 mice is responsible for eosinophil accumulation in the heart. This is consistent with our previous findings that NK cell depletion results in increased eotaxin expression and eosinophil infiltration in the heart<sup>187</sup>. We only observed these differences between  $\Delta$ dblGATA1 and IFN $\gamma$ <sup>-/-</sup>IL-17A<sup>-/-</sup> $\Delta$ dblGATA1 mice in the heart. The intestine showed efficient CCR3<sup>+/+</sup> eosinophil trafficking in both recipients. This suggests that the severe, Th2-driven EAM developing in the absence of IFN $\gamma$  and IL-17A is necessary for high cardiac eotaxin expression and eosinophilic myocarditis.

We identified the cellular sources of eotaxins in the heart. CCL11 was produced by interstitial CD140a<sup>+</sup> cardiac fibroblasts and was detectable in  $\Delta$ dblGATA1 and IFN $\gamma$ <sup>-/-</sup>IL-17A<sup>-/-</sup> $\Delta$ dblGATA1 mice. While not studied in the heart, CCL11 expression in experimental asthma was found in the peribronchial and perivascular regions, perhaps consistent with expression by fibroblasts, and in areas of severe inflammation<sup>72</sup>. In DSS-induced colitis, intestinal macrophages expressed CCL11<sup>73</sup>. Using immunohistochemistry, we detected CCL24<sup>+</sup> cells only in the hearts of immunized IFN $\gamma$ <sup>-/-</sup>IL-17A<sup>-/-</sup> $\Delta$ dblGATA1 mice where they localized to inflammatory foci. We identified these cells as F4/80<sup>+</sup> macrophages. A previous study noticed *Ccl24* expression by microarray in CX3CR1<sup>+</sup> cardiac macrophages, although other cell types were not analyzed<sup>202</sup>. In experimental asthma, CCL24 was expressed in the same areas as CCL11 and in bronchoalveolar lavage fluid<sup>72</sup>. Thus, multiple cell types are capable of CCL11 and/or CCL24 expression and differ between organs.

Our *in vitro* experiments showed that cardiac fibroblasts expressed CCL11 and, to a much lesser extent, CCL24 in response to IL-4 or IL-13 stimulation. Macrophages responded to IL-4 and IL-13 stimulation with CCL24 expression; CCL11 was not induced. The ability of IL-4 and IL-13 to elicit eotaxins is known<sup>203-205</sup>, but has not been tested in cardiac fibroblasts. We speculate that T cells and type 2 innate lymphoid cells are major producers of IL-4 and IL-13, respectively and control eotaxin production in the heart.

*Ccl24* is upregulated over 100-fold while *Ccl11* is upregulated 20-fold in EAM, suggesting that CCL24 may be more important for eosinophil trafficking to the heart. This would be similar to allergic lung inflammation, in which eosinophil trafficking is controlled by CCL24<sup>72</sup> but different from the intestine and thymus, in which eosinophil trafficking is regulated by CCL11<sup>69</sup>. The relative importance of CCL11 versus CCL24 in the heart remains to be determined.

Analyzing endomyocardial biopsies, we found that CCL11 and CCL26 expression was increased in patients with eosinophilic myocarditis compared to chronic lymphocytic myocarditis (17-fold and 47-fold, respectively) and was positively correlated with the number of infiltrating eosinophils. We conclude that eosinophil trafficking to the heart in myocarditis patients likely occurs in response to eotaxin signaling. We speculate that CCL11 and CCL26 may both recruit eosinophils to the heart. An upregulation of CCL11 and CCL26 is also observed in patients with atopic dermatitis<sup>206-208</sup>, while only CCL26 is upregulated in eosinophilic esophagitis and asthma<sup>209, 210</sup>.

Eotaxin expression in the heart has not been studied in the context of disease. In healthy human heart tissue CCL11 and CCL26, but not CCL24, were detected by northern blot<sup>75-77</sup>. CCL24 mRNA was detectable in 2/11 eosinophilic myocarditis biopsies but none of the chronic lymphocytic myocarditis biopsies. However, the mRNA expression level of CCL24 was much lower than that of CCL11 and CCL26 and, thus, may not be important. In this aspect, the mouse model of myocarditis, where CCL11 and CCL24 are highly upregulated, differs from the patient samples. We did not determine eotaxin protein levels in the endomyocardial biopsies. Follow-up studies should measure eotaxin in the heart and serum.

The cellular source and regulation of eotaxins has not been studied in the human heart. In ulcerative colitis, CCL26 was expressed in intestinal nerve ganglia<sup>211</sup> and CCL11 in intestinal macrophages and epithelial cells<sup>73</sup>. In EGPA, CCL26 was detected in endothelial cells, nasal epithelium, and other cells<sup>212</sup>. In asthmatics, CCL26 is expressed in bronchial epithelial cells<sup>209</sup>. Similar to our observations in the mouse, eotaxin-production in various human cell types is induced by IL-4 and/or IL-13<sup>203-205, 207, 209, 213, 214</sup>. Depending on the cell type, this stimulation results in CCL11, CC24, or CCL26 production. This pathway likely also regulates eotaxin production in the human heart during myocarditis.

In this study, we demonstrate the importance of eotaxins and CCR3 for eosinophil localization to the heart. Several therapeutics blocking this pathway are being evaluated in allergic diseases<sup>215-217</sup>, where they decrease tissue eosinophilia. Ongoing trials are assessing CCL11 blockade in the eosinophilic autoimmune diseases ulcerative colitis and bullous pemphigoid (ClinicalTrials.gov identifiers: NCT01671956, NCT02226146). However, none of the treatments have been studied in eosinophilic myocarditis, HES or EGPA. The potential of targeting eotaxins or CCR3 to prevent eosinophil-mediated heart damage remains to be tested.

## Chapter 4: Eosinophil-derived IL-4 drives heart failure

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Diny NL, Baldeviano GC, Talor MV, Barin JG, Ong S, Bedja D, Hays AG, Gilotra NA, Coppens I, Rose NR, Čiháková D. Eosinophil-derived IL-4 drives progression of myocarditis to inflammatory dilated cardiomyopathy. *J Exp Med*. 2017 Mar 16. [Epub ahead of print]. (Copyright: the authors.)

### Summary

Inflammatory dilated cardiomyopathy (DCMi) is a major cause of heart failure in children and young adults. DCMi develops in up to 30% of myocarditis patients, but the mechanisms involved in disease progression are poorly understood. Patients with eosinophilia frequently develop cardiomyopathy. We used the experimental autoimmune myocarditis (EAM) model to determine the role of eosinophils in myocarditis and DCMi. Eosinophils were dispensable for myocarditis induction but required for progression to DCMi. Eosinophil-deficient  $\Delta$ dblGATA1 mice, in contrast to WT mice, showed no signs of heart failure by echocardiography. Induction of EAM in hypereosinophilic IL-5Tg mice resulted in eosinophilic myocarditis with severe atrial inflammation, which progressed to severe DCMi. This was not a direct effect of IL-5 as IL-5Tg $\Delta$ dblGATA1 mice were protected from DCMi while IL-5<sup>-/-</sup> mice exhibited DCMi comparable to WT mice. Eosinophils drove progression to DCMi through their production of IL-4. Our experiments showed eosinophils were the major IL-4 expressing cell type in the heart during EAM, IL-4<sup>-/-</sup> mice were protected from DCMi like  $\Delta$ dblGATA1 mice, and eosinophil-specific IL-4 deletion resulted in improved heart function. In conclusion, eosinophils drive progression of myocarditis to DCMi, cause severe DCMi when present in large numbers, and mediate this process through IL-4.



## Introduction

Myocarditis, the inflammation of the heart muscle in the absence of an ischemic event, can cause sudden cardiac death and heart failure<sup>5, 12</sup>. While most myocarditis patients recover from the acute illness, a major burden lies in the sequela inflammatory dilated cardiomyopathy (DCMi). About 9-16% of patients with new onset DCMi have evidence of prior myocarditis<sup>48, 49, 218</sup>, yet the mechanisms involved in disease progression are poorly understood<sup>219</sup>. DCMi is the major cause of heart failure in patients under 40 years of age. The 5-year survival rate is less than 50%<sup>138, 173</sup>. Currently, the only option for patients with end-stage DCMi is heart transplantation. Even if a transplant is available, patients with a history of myocarditis have a particularly poor survival rate<sup>174</sup>. No drugs are available to arrest or delay development of DCMi or to improve long-term survival. Thus, there is an urgent need to better understand the progression of myocarditis to DCMi and to develop new therapeutic approaches to prevent it.

Eosinophils play an important role in heart disease. Cardiac complications occur in about 20-50% of patients with prolonged elevation of eosinophil numbers, such as hypereosinophilic syndrome (HES) and eosinophilic granulomatosis with polyangiitis, and are a major cause of mortality<sup>32-35, 182</sup>. Predominant infiltration of the heart with eosinophils characterizes the clinically-recognized subtype eosinophilic myocarditis. In addition to HES and eosinophilic granulomatosis with polyangiitis patients, eosinophilic myocarditis can develop in patients with parasitic infections, in response to toxins or drugs, or by unknown etiology<sup>14, 24</sup>. Eosinophilic myocarditis is rarely diagnosed clinically but is found in up to 0.5% of patients in a hospital autopsy series<sup>153</sup> and about 7% of explanted hearts from transplant patients<sup>152-154</sup>. It is not known at what rate eosinophilic myocarditis patients progress to DCMi or how this rate compares against other myocarditis subtypes.

Eosinophils infiltrate the site of inflammation and release preformed toxic granule proteins, cytokines, and growth factors, thereby contributing to tissue injury and remodeling<sup>67</sup>. Eosinophils have been thought to activate other cardiac cell types, to be directly cytotoxic to the endocardium or cardiomyocytes, or to

release granule proteins or pro-thrombotic factors <sup>39, 43-46, 186</sup>. However, many of these studies are purely observational and the proposed pathogenic mechanisms have not been thoroughly tested. Others have demonstrated eosinophilic myocarditis developing after infection with various parasites <sup>220-225</sup> or spontaneously in DBA/2 mice <sup>226-228</sup>, Socs1-deficient mice<sup>229</sup>, and Bcl6-deficient mice <sup>230</sup>. However, none of these studies have examined the importance of eosinophils for myocarditis severity, their role in progression to DCMi, or how eosinophils damage the heart. The NIH workshop report on the Research Needs of Eosinophil-Associated Diseases points out an urgent need for preclinical models and mechanistic understanding of eosinophil-mediated cardiac damage <sup>184</sup>.

To study myocarditis and DCMi, our laboratory has developed an animal model of experimental autoimmune myocarditis (EAM) <sup>58, 60</sup>. Mice lacking both the key T helper 1 (Th1) and Th17 cytokines IFN $\gamma$  and IL-17A developed a rapidly fatal eosinophilic myocarditis after induction of EAM. Ablation of eosinophils in these mice improved survival <sup>189</sup>. In another model, natural killer cell depletion resulted in increased eosinophil infiltration in the heart, aggravating myocarditis <sup>187</sup>. These results suggest that eosinophils are highly pathogenic in myocarditis and DCMi. In this study, we used eosinophil-deficient and hypereosinophilic mouse models to examine the impact of eosinophils in myocarditis and its sequela DCMi. Moreover, we identified the mechanisms by which eosinophils contribute to pathology.

## **Materials and Methods**

### **Patients**

Patients with endomyocardial biopsy (EMB)-confirmed eosinophilic myocarditis for whom cardiac magnetic resonance imaging was also available from 2000-2014 were retrospectively identified at the Johns Hopkins Hospital, Department of Cardiology. EMB of the right ventricular septum was performed using the Argon Jawz Endomyocardial Bioptome. A cardiac pathologist examined all specimens at a minimum of four section levels with typical stains <sup>105</sup>. Diagnosis of eosinophilic myocarditis was established using the Dallas Criteria

for myocarditis and presence of eosinophilic infiltrate as detected on H&E-stained sections<sup>1</sup>. Patients underwent clinically indicated cardiac magnetic resonance with a 1.5-T scanner (Siemens Healthcare, Avanto, Erlangen, Germany) including standard cine and late gadolinium enhancement studies covering the whole heart. Analysis was performed on a dedicated workstation by an experienced observer. The study was approved by the Johns Hopkins Institutional Review Board.

## **Mice**

IL-5Tg (founder line NJ.1638)<sup>195</sup> and EoCre mice<sup>231</sup> were kindly provided on the BALB/c background by Drs. James and Nancy Lee, Mayo Clinic, Scottsdale. IL-5<sup>-/-</sup> mice<sup>232</sup> were kindly provided by Dr. Marc Rothenberg. BALB/cJ wildtype (WT, Jackson Laboratories Stock #651),  $\Delta$ dblGATA1 (<sup>194</sup>, #5653), IL-4<sup>-/-</sup> (<sup>233</sup>, #2496), 4get (<sup>234</sup>, #4190), IL-4/13<sup>flox/flox</sup> (<sup>235</sup>, #15859), and ROSA-DTA (<sup>236</sup>, #9670) mice were purchased from Jackson Laboratories. All mice were on the BALB/c background. Mice were housed in specific pathogen-free animal facilities at the Johns Hopkins University. Experiments were conducted on 6–10-weeks-old age-matched male mice in compliance with the Animal Welfare Act and the principles set forth in the Guide for the Care and Use of Laboratory Animals. All methods and protocols were approved by the Animal Care and Use Committee of Johns Hopkins University.

## **Induction of EAM**

To induce EAM, mice received subcutaneous immunizations on days 0 and 7 of 100  $\mu$ g myosin heavy chain- $\alpha$  (MyHC)<sub>614-629</sub> peptide (Ac-SLKLMATLFSTYASAD, Genscript)<sup>237</sup> emulsified in CFA (Sigma-Aldrich) supplemented to 5 mg/ml heat-killed *Mycobacterium tuberculosis* strain H37Ra (Difco). On day 0, mice also received 500 ng pertussis toxin intraperitoneally (List Biologicals)<sup>60</sup>.

## **Assessment of EAM severity and fibrosis**

Heart tissue was fixed in SafeFix solution (Thermo Fisher Scientific), embedded, and cut into 5- $\mu$ m serial sections. Ventricular inflammation was scored by microscopic assessment of the area of infiltration with hematopoietic cells on H&E stained sections according to the following scale: grade 0, no inflammation;

grade 1, <10% of the heart section is infiltrated; grade 2, 10-30%; grade 3, 30-50%; grade 4, 50-90%; grade 5, >90%. Ventricular fibrosis was scored on Masson's trichrome-stained sections according to the same scale. Atrial inflammation and fibrosis was scored on the following scale: none (0), mild (1), moderate (2), severe (3). Grading was performed by two independent, blinded investigators and averaged <sup>60</sup>.

### **Light microscopy**

Images were acquired on a Olympus BX43 microscope with a Olympus DP72 camera using cellSens Standard version 1.4.1 (Olympus).

### **Electron microscopy**

Mice were anesthetized, the heart was removed, and the left ventricle was cut into 2-3mm<sup>3</sup> pieces and immediately fixed in 2.5% glutaraldehyde (EM grade, Electron Microscopy Sciences, Hatfield, PA) dissolved in 0.1M Na cacodylate (pH7.4) for 2h at room temperature. Samples were processed as described <sup>238</sup> before examination with a Philips CM120 Electron Microscope (Eindhoven, the Netherlands) under 80 kV. Images were acquired with AMT Image Capture Engine V602 (Advanced Microscopy Techniques).

### **Echocardiography**

Transthoracic echocardiography was performed using the Acuson Sequoia C256 ultrasonic imaging system (Siemens) with a 13 MHz transducer. Conscious, depilated mice were held in a supine position. The heart was imaged in the two-dimensional mode in the parasternal short axis view. An M-mode cursor was positioned perpendicular to the interventricular septum and the left ventricular posterior wall at the level of the papillary muscles. The LVEDD, LVESD, interventricular septal wall thickness at end-diastole, and LV posterior wall thickness at end-diastole were measured three times for each mouse from a frozen M-mode tracing and averaged. EF, RWT, and LV mass were calculated from these parameters as previously described

<sup>239</sup>.

### **Flow cytometry**

For flow cytometry analysis, single cell suspensions were made from mouse spleen by mechanical dissociation followed by red blood cell lysis with ACK lysis buffer (Quality Biologicals). Heart-infiltrating leukocytes were isolated by perfusing mouse hearts for 3 min with PBS 0.5% FBS, and digested for 30 min at 37°C in gentleMACS C Tubes (Miltenyi Biotec) with 3000 U/ml Collagenase II and 90 U/ml DNase I (Worthington). For intracellular cytokine staining, cells were stimulated *in vitro* for 5h with 50 ng/ml phorbol 12-myristate 13-acetate, 750 ng/ml ionomycin (Sigma), GolgiStop, and GolgiPlug (BD Biosciences) prior to staining. Viability was determined by LIVE/DEAD staining according to manufacturer's instructions (Life Technologies). For intracellular cytokine staining, cells were resuspended in Cytofix/Cytoperm (BD Biosciences). Cells were blocked with anti-CD16/CD32 (eBioscience), and were stained with fluorochrome-conjugated monoclonal antibodies (eBioscience, BD Biosciences, BioLegend). For absolute quantification, viable cells were counted with Trypan blue or using CountBright beads (Thermo Fisher Scientific). Samples were acquired on an LSR II cytometer running FACSDiva 6 (BD Biosciences). Data were analyzed with FlowJo 10 (TreeStar).

### **Isolation of primary adult mouse cardiomyocytes**

To isolate cardiomyocytes and cardiac fibroblasts, the heart was dissected from 6–8-wk-old male mice pretreated with heparin as previously described<sup>197</sup>. The aorta was cannulated, and the heart was perfused with calcium-free perfusion buffer, and digested with 3000 U/ml collagenase II (Worthington) and 0.05 mg/ml Protease XIV (Sigma-Aldrich) for 15 min at 30°C followed by mechanical dissociation. Cardiomyocytes were separated from resulting suspensions by their rapid spontaneous precipitation. Isolated CMs were cultured in mouse laminin-coated plates. Non-adherent cells were washed off after 1h.

### **Hydroxyproline assay**

Heart samples were weighed, homogenized in de-ionized water, and hydrolyzed overnight in 6N HCl at 120°C. Lysates were transferred to and desiccated in 96-well plates, and reconstituted in de-ionized water.

After incubation with 50 mM Chloramine T (Sigma-Aldrich) followed by 1 M dimethylaminobenzaldehyde (Sigma-Aldrich), the OD values were read at 570 nm. The concentration of hydroxyproline was determined by a 1–100 µg/ml standard curve of hydroxyproline (Sigma-Aldrich) and normalized to starting heart tissue mass.

### **ELISA**

Sera were stored at -80°C prior to analysis. IL-5 was determined by quantitative sandwich ELISA according to manufacturers' recommended protocols (R&D Systems). For anti-myosin IgG ELISA, plates were coated with 0.5 µg/ml MyHC<sub>614-629</sub> peptide (Ac-SLKLMATLFSTYASAD, Genscript) overnight at 4°C, washed with 1X PBS 2% FBS, and incubated with sera for 2h at room temperature. Plates were washed and incubated with secondary anti-mouse IgG antibody (Abcam) diluted 1:1000 in 1XPBS for 2h at room temperature. Plates were washed and developed with Alkaline Phosphatase (Biorad) and OD was read at 405 nm.

### **Quantitative PCR**

Tissue RNA was extracted in TRIzol (Invitrogen) and quantitated. cDNA was synthesized with the High Capacity cDNA Reverse Transcription kit (Life Technologies), amplified with iQ SYBR Green Mastermix (Bio-Rad), and acquired on the MyiQ2 thermocycler (Bio-Rad) running iQ5 software (Bio-Rad). Data were analyzed by the 2- $\Delta\Delta C_t$  method<sup>198</sup>, normalizing threshold cycles first to Gapdh or Hprt expression, and then to controls. Primer sequences are listed in Table 7.

### **Statistics**

Two groups with normally distributed data were analyzed using student's t-test. Mann-Whitney test was used for nonparametric data. Multiple group analysis was performed by one-way ANOVA followed by Tukey's multiple comparisons test for continuous variables or by Kruskal-Wallis test followed by Dunn's multiple comparisons test for nonparametric data. Calculations were performed in Prism 6 (GraphPad Software Inc.). *P* values <0.05 were considered statistically significant and are denoted by asterisk: \**p*<0.05, \*\**p*<0.01, \*\*\**p*<0.001.

**Table 7: Primer sequences**

| Gene          | Forward primer sequence (5'-3') | Reverse primer sequence (5'-3') |
|---------------|---------------------------------|---------------------------------|
| <i>Ccl11</i>  | gaatcaccaacaacagatgcac          | tcctggacccacttcttctt            |
| <i>Ccl24</i>  | tcttagggcccttcttggtg            | aattccagaaaaccgagtgg            |
| <i>Col1a2</i> | agcaggtccttggaacctt             | aaggagtttcatctggccct            |
| <i>Col3a1</i> | gtgaacggggcgaagctggtt           | gcggctcctggaagcccatttg          |
| <i>Gapdh</i>  | tcctcctcagaccgctttt             | tctgtggagtgcccttg               |
| <i>Gata3</i>  | ctcgggcattcgatcatggaa           | ggatacctctgcaccgtagc            |
| <i>Hprt</i>   | tcagtcaacgggggacataaa           | ggggctgtactgttaaccag            |
| <i>Mmp2</i>   | tttgctcgggccttaaaagtat          | ccatcaaacgggtatccatctc          |
| <i>Mmp9</i>   | tgccatttcgacgacgac              | gtgcaggccgaataggagc             |
| <i>Rorc</i>   | atcctgtaatggcttggtggg           | tcaaccagcaccagacagag            |
| <i>Tbx21</i>  | atcctgtaatggcttggtggg           | tcaaccagcaccagacagag            |
| <i>Timp1</i>  | gcaactcggacctggtcataa           | cggcccgatgatgagaaact            |
| <i>Timp2</i>  | cttctgcaactccgacatcgt           | ggggcatcttactgaagcctc           |

## Results

### Eosinophil-deficient mice develop myocarditis but are protected from dilated cardiomyopathy

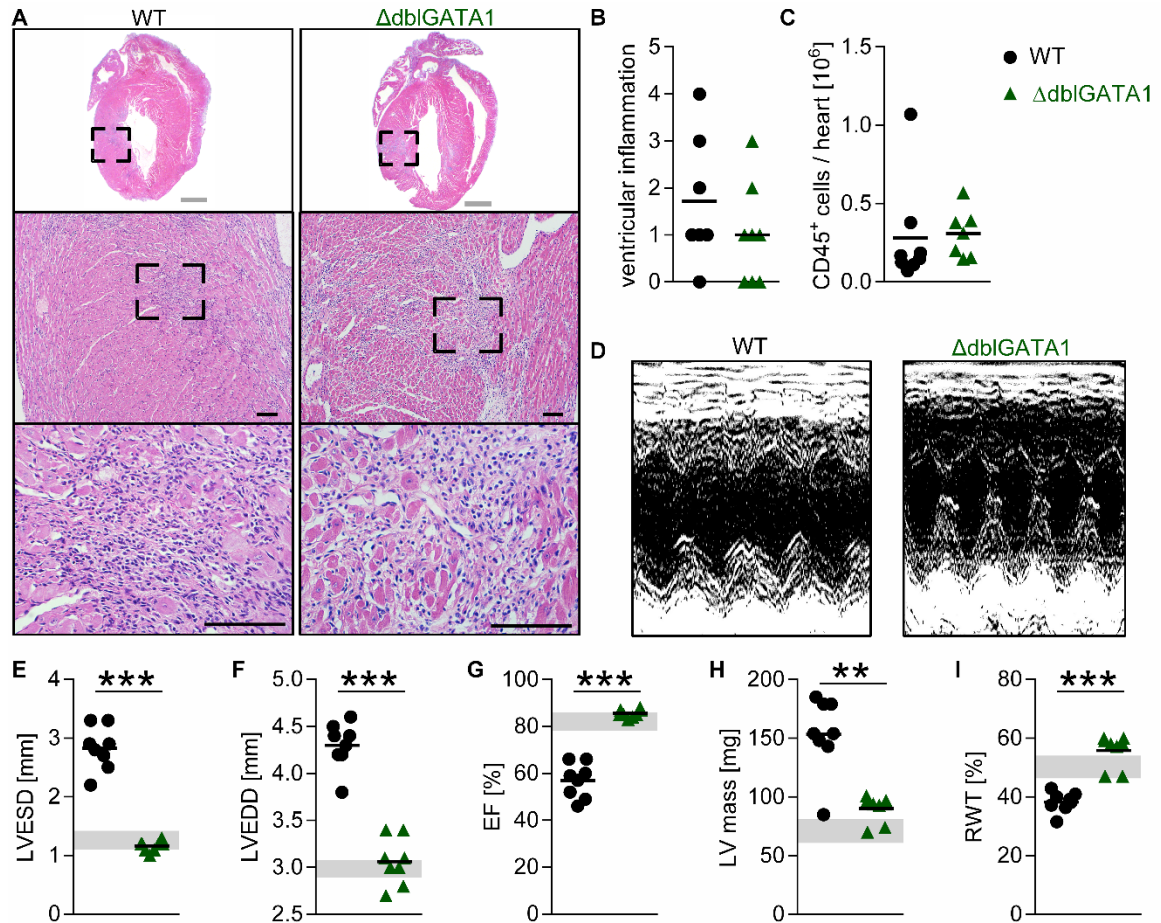
To study the mechanism of eosinophil-mediated damage in myocarditis and its sequela DCMi, we used eosinophil-deficient  $\Delta$ dblGATA1 mice. In these mice, a deletion in the promoter of *Gata1* blocks eosinophil development in the bone marrow<sup>194</sup>. Myocarditis was induced in wildtype (WT) and  $\Delta$ dblGATA1 mice and the degree of inflammation was compared on day 21 of EAM.  $\Delta$ dblGATA1 mice developed myocarditis with similar severity as WT mice (Figure 18A, B). Moreover, no differences were observed in the number of heart-infiltrating CD45<sup>+</sup> cells by flow cytometry at day 21 of EAM (Figure 18C).

In WT mice, EAM progresses to DCMi<sup>12</sup>. Surprisingly, typical signs of ventricular dilation were present in WT, but not  $\Delta$ dblGATA1 mice examined by echocardiography (Figure 18D).  $\Delta$ dblGATA1 mice had preserved left ventricular (LV) end-diastolic (LVEDD) and end-systolic (LVESD) diameters and preserved ejection fraction (EF) at day 42 of EAM (Figure 18E-G).  $\Delta$ dblGATA1 mice were also protected from the increase in LV mass and thinning of the LV wall (relative wall thickness, RWT) seen in WT mice (Figure 18H-I). These results indicate that eosinophils are not required for EAM development, but are necessary for progression of myocarditis to DCMi.

#### **$\Delta$ dblGATA1 mice develop fibrosis but show altered tissue remodeling**

Progression to DCMi is associated with changes in the connective tissue in the heart, namely fibrosis and tissue remodeling. We previously reported on EAM in IL-17A<sup>-/-</sup> and IL-17RA<sup>-/-</sup> mice, in which protection from DCMi is associated with reduced fibrosis<sup>197, 240</sup>.  $\Delta$ dblGATA1 mice, however, developed fibrosis to a similar degree as WT mice. Fibrosis was assessed by scoring of Masson's trichrome stained heart sections (Figure 19A) and quantitation of total cardiac hydroxyproline, a collagen-specific amino acid (Figure 19B). These results demonstrate that heart function can be preserved despite development of cardiac fibrosis. Protection of IL-17A<sup>-/-</sup> mice from DCMi is associated with differential expression of *Col1a2*, *Col3*, *Timp1*, and *Mmp9*<sup>240</sup>. Expression of these genes was not changed in  $\Delta$ dblGATA1 mice (Figure 19C). However, other tissue remodeling-associated genes, *Mmp2* and its regulator *Timp2*, were decreased in  $\Delta$ dblGATA1 mice (Figure 19D). This indicates that eosinophils promote DCMi through pathways that are identifiably distinct from those driven by IL-17 signaling. High serum MMP2 levels are associated with a poor prognosis in acute and chronic heart failure<sup>241, 242</sup>, suggesting that similar processes may be involved in patients with heart failure.



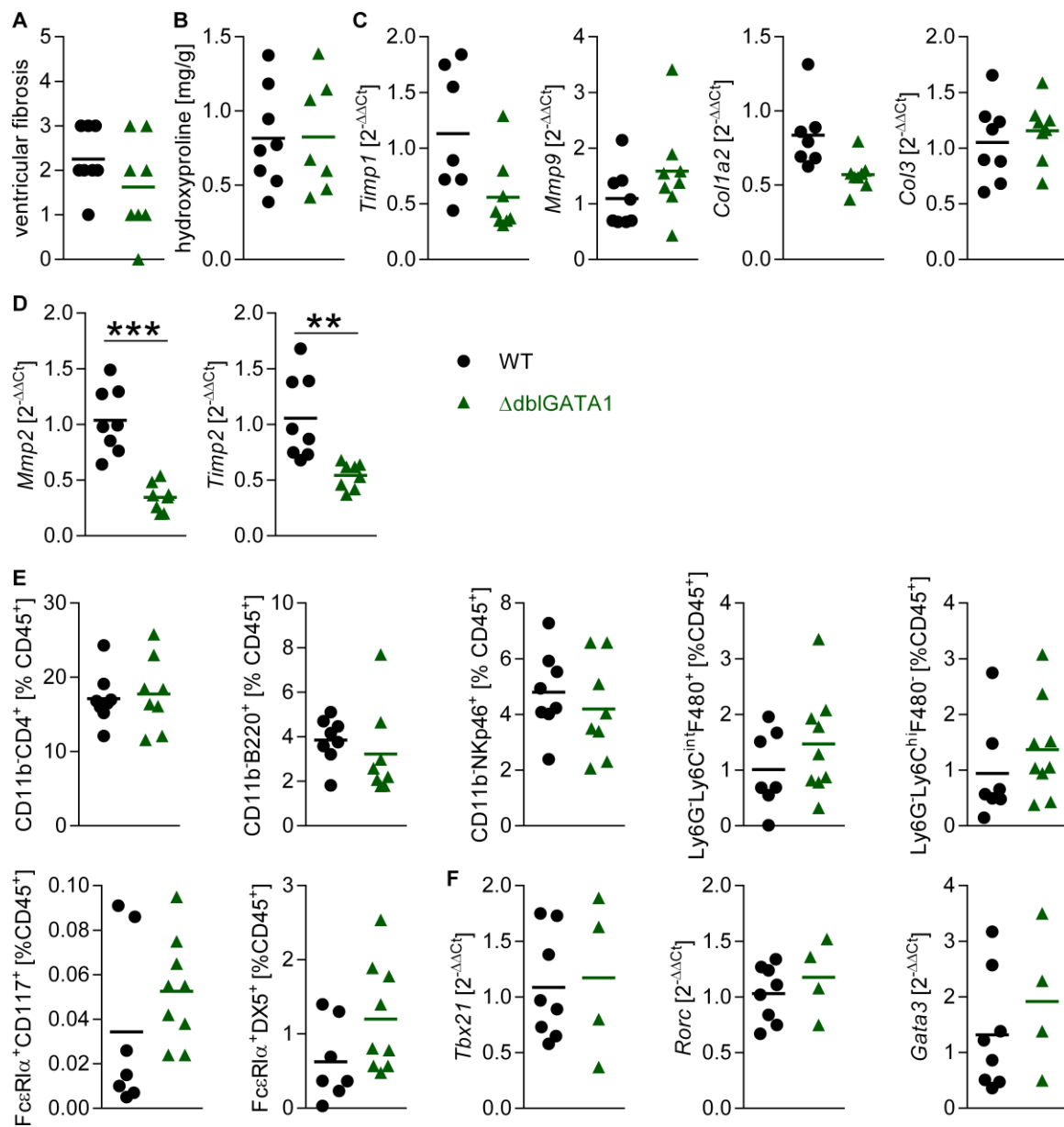


**Figure 18: Eosinophil-deficient mice develop EAM, but are protected from DCMi**

A) Representative images of H&E stained heart sections from day 21 of EAM. B) The area of infiltration on H&E stained heart sections was scored on day 21 of EAM (C) Total heart-infiltrating CD45<sup>+</sup> cells at day 21 of EAM were determined by flow cytometry (D-I) Echocardiography in WT and  $\Delta$ dblGATA1 mice on day 45 of EAM D) Representative M-mode pictures. E-I) Grey bands indicate 95% confidence interval for naïve WT mice. B, C, E-I) Groups were compared using t-test. Data are representative of 3-5 independent experiments with 6-8 mice/group. Grey scale bars: 1mm, black scale bars: 100 $\mu$ m. Asterisk indicate significance: \*\*p<0.01, \*\*\*p<0.001.

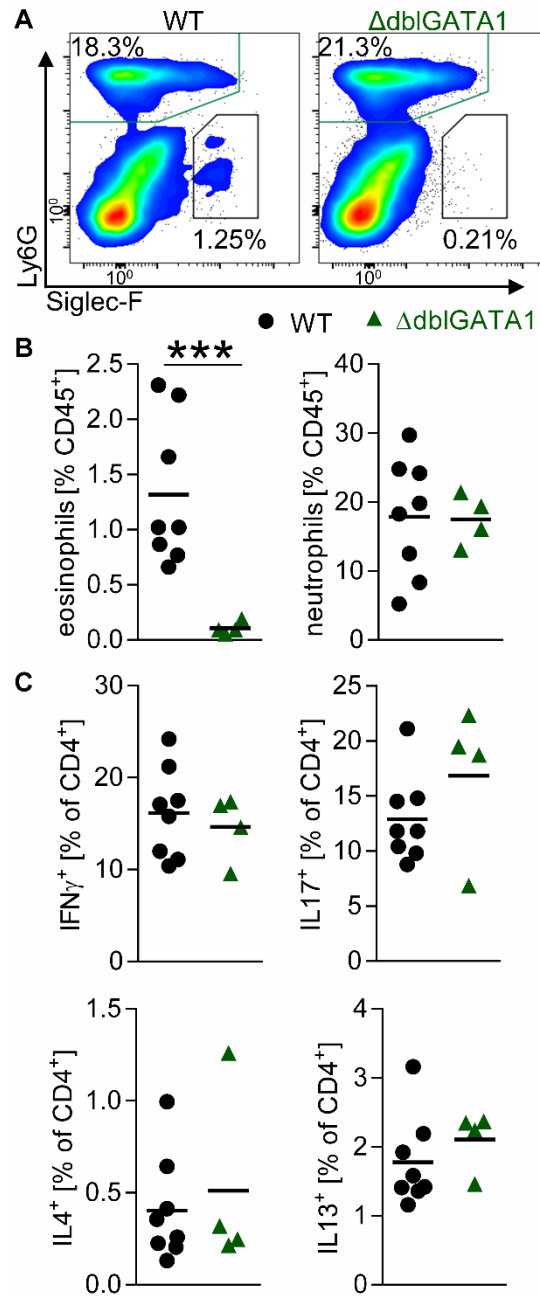
**Figure 19: Protection of  $\Delta$ dbpGATA1 mice from DCMi is accompanied by changes in tissue remodeling-associated genes not changes in heart-infiltrating cell populations**

A-D) Mice were analyzed at day 45 of EAM. A, B) The extent of fibrosis was determined by scoring fibrotic areas on histological sections from 5 experiments (A) and by quantifying the amino acid hydroxyproline in heart samples from 2 experiments (B). C, D) Gene expression in heart homogenates was analyzed by qPCR. Data are representative of 2-3 independent experiments with 5-8 mice/group. E) Frequency of heart infiltrating cells was determined by flow cytometry on day 21 of EAM. Data are combined from different experiments. Cell types were gated as follows: neutrophils: CD11b<sup>+</sup>Ly6G<sup>+</sup>, T cells: CD11b<sup>-</sup>CD3<sup>+</sup>CD4<sup>+</sup> or CD8<sup>+</sup>, B cells: CD11b<sup>-</sup>B220<sup>+</sup>, NK cells: CD11b<sup>-</sup>CD3<sup>+</sup>NKp46<sup>+</sup>, monocytes / macrophages: CD11b<sup>+</sup>Ly6G<sup>-</sup>Ly6C<sup>+</sup>, basophils: CD11b<sup>+</sup>Ly6G<sup>-</sup>Ly6C<sup>+</sup>FcεRIα<sup>+</sup>DX5<sup>+</sup>, mast cells: CD11b<sup>+</sup>Ly6G<sup>-</sup>Ly6C<sup>+</sup>FcεRIα<sup>+</sup>CD117<sup>+</sup>. F) Expression of transcription factors was determined by quantitative PCR from heart homogenates from day 21 of EAM and normalized to expression of CD3 and WT controls. Data are representative of 2 independent experiments with 4-8 mice/group. Groups were compared by Mann-Whitney test (A) or t-test (B-F). Asterisk indicate significance: \*\*p<0.01, \*\*\*p<0.001.



### **Lack of eosinophils does not alter cardiac inflammatory infiltrate in EAM**

In WT mice, eosinophils accounted for 1-3% of heart-infiltrating cells at day 21, the peak of EAM (Figure 20A, B). As expected, eosinophils were not detected in  $\Delta$ dblGATA1 mice (Figure 20A, B). We first hypothesized that eosinophils may affect the makeup of the cellular infiltrate in myocarditis. However, the lack of eosinophils in  $\Delta$ dblGATA1 mice did not affect the composition of heart-infiltrating cell populations (Figure 20B, Figure 19E). We did not observe any differences in the percentage of heart-infiltrating neutrophils, T cells, B cells, NK cells, monocytes / macrophages, basophils, or mast cells. Production of IL-17A, IFN $\gamma$ , IL-4, and IL-13 by heart-infiltrating T helper cells was comparable between WT and  $\Delta$ dblGATA1 mice (Figure 20C, Figure 19F), indicating that the lack of eosinophils did not affect T cell polarization. These findings are consistent with the notion that the lack of eosinophils conferred disease protection in  $\Delta$ dblGATA1 mice.

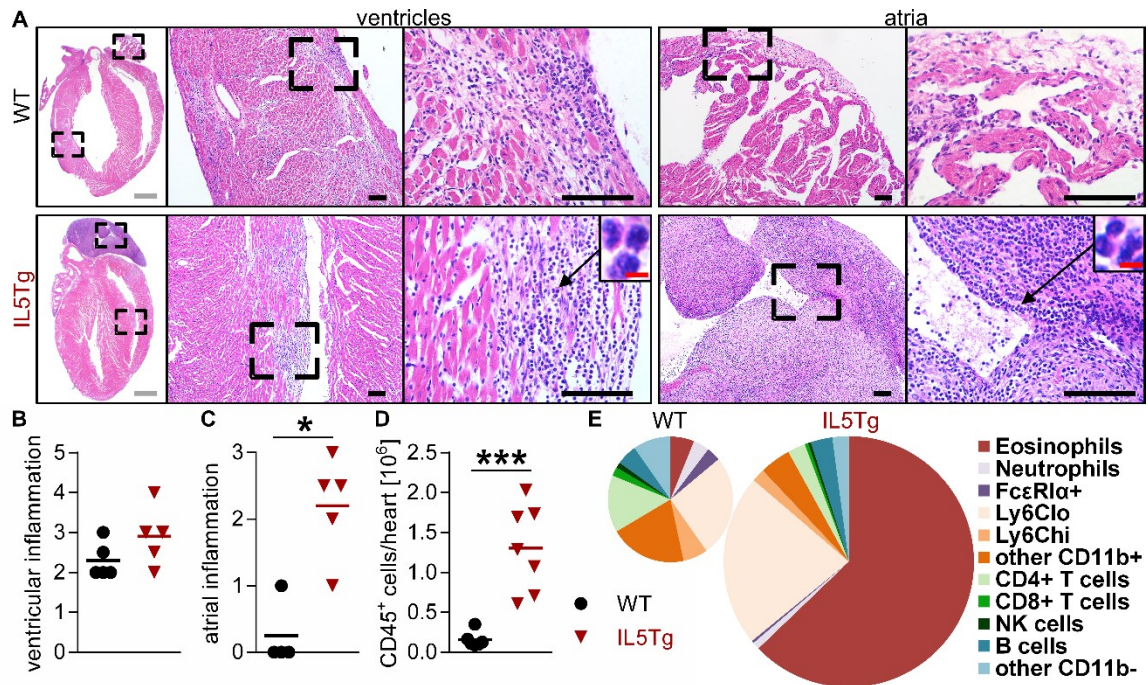


**Figure 20: Lack of eosinophils does not alter inflammatory infiltrate in EAM**

A-C) Frequency of heart-infiltrating cells was determined by flow cytometry on day 21 of EAM. A) Flow plots show viable CD45<sup>+</sup> cells. C) Cytokine production by heart-infiltrating CD4<sup>+</sup> T cells. Data are representative of 2-5 independent experiments with 4-8 mice/group. Groups were compared by t-test. Asterisk indicate significance: \*\*\*p<0.001.

### **IL-5Tg mice develop eosinophilic myocarditis**

To address whether increased levels of eosinophils would aggravate myocarditis and DCMi, we used transgenic mice expressing IL-5 under the CD3 promoter (IL-5Tg). These mice express high serum levels of IL-5, a key cytokine for eosinophilopoiesis and eosinophil survival, and develop extensive peripheral eosinophilia<sup>195</sup>. Following immunization, IL-5Tg mice developed severe eosinophilic myocarditis (Figure 21A). Scoring of the degree of inflammation revealed a trend towards more severe inflammation in the ventricles (Figure 21A, B) and significantly more severe inflammation in the atria of IL-5Tg mice (Figure 21A, C). This resulted in an increased number of heart-infiltrating CD45<sup>+</sup> cells in IL-5Tg compared to WT mice (Figure 21D). As evident from the histology, eosinophils comprised a large proportion of heart-infiltrating cells (Figure 21A, high magnification inserts). We quantified heart-infiltrating cell types using flow cytometry. Eosinophils accounted for over 60% of CD45<sup>+</sup> cells in the hearts of IL-5Tg mice compared to only 3% in WT mice (Figure 21D). This corresponded to a dramatic difference in absolute eosinophil numbers in both atria and ventricles (Figure 22A). Due to this tremendous increase in heart-infiltrating eosinophils and overall increase of CD45<sup>+</sup> cells, the absolute numbers of other cell populations were either not different or higher in IL-5Tg mice (Figure 22B). A possible explanation for the severe eosinophilic atrial inflammation was increased expression of eotaxins in the atria of IL-5Tg mice (Figure 22C). Eotaxins are key chemokines for eosinophil trafficking to the heart<sup>74</sup>. Taken together, these results show that IL-5Tg mice develop severe eosinophilic myocarditis. EAM in IL-5Tg mice is a novel inducible model of eosinophilic myocarditis.



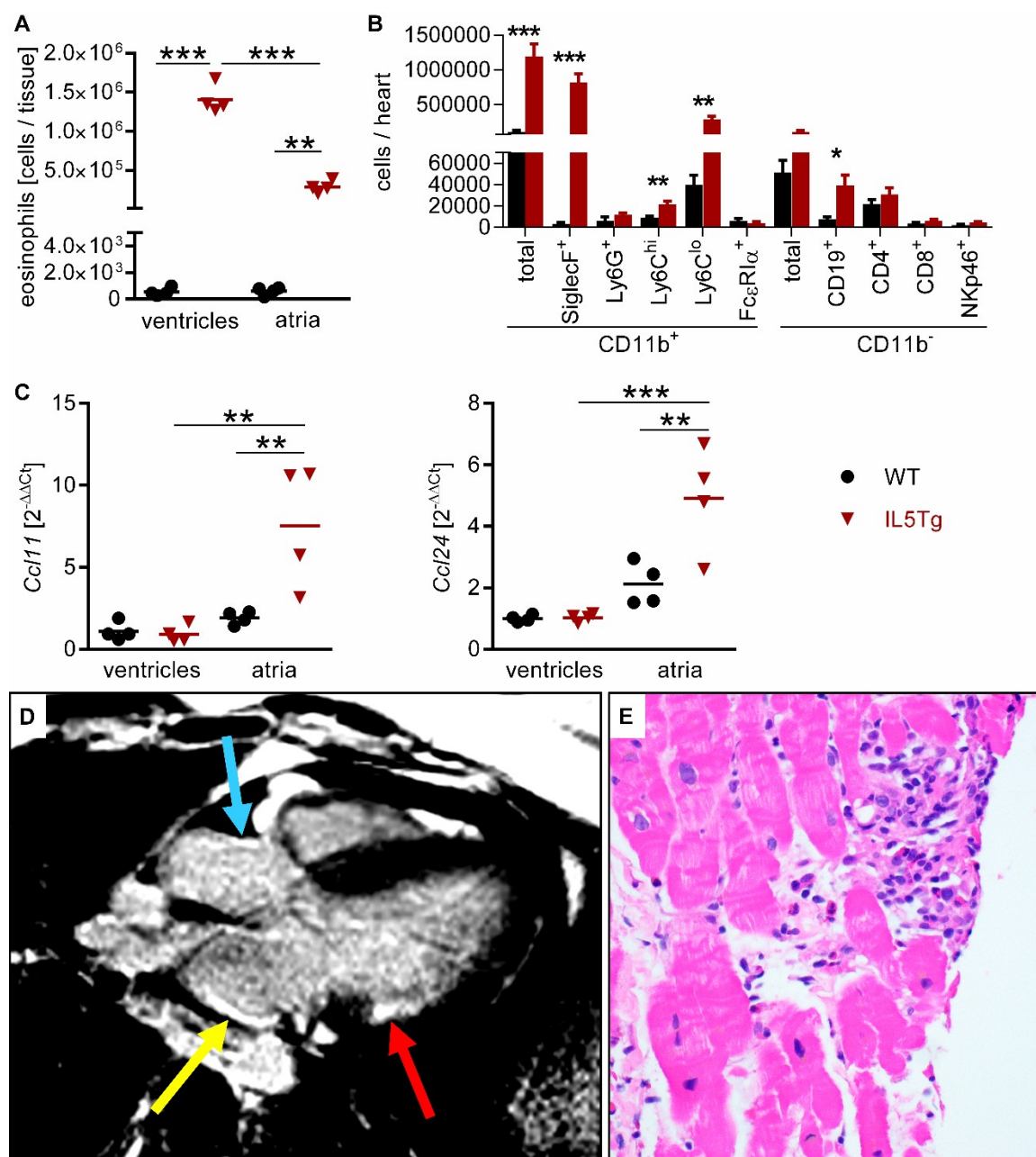
**Figure 21: IL-5 transgenic mice develop severe eosinophilic myocarditis**

A) Representative images of H&E stained heart sections from day 21 of EAM. B, C) Scoring of inflammation in the ventricles (B) and atria (C). D, E) Total heart-infiltrating CD45<sup>+</sup> cells (D) and heart-infiltrating cell populations (E) were quantified by flow cytometry. Data are representative of 2-3 independent experiments with 4-5 mice/group. Groups were compared using Mann-Whitney test (B, C) or t-test (D). Grey scale bars: 1mm, black scale bars: 100µm. Asterisk indicate significance: \*p<0.05, \*\*\*p<0.001.

**Figure 22: Atrial versus ventricular inflammation in IL-5Tg mice and an eosinophilic myocarditis patient**

A-C) Mice were analyzed at day 21 of EAM. A) Gene expression in ventricles and atria was determined by qPCR and normalized to Gapdh. B, C) Absolute numbers of heart-infiltrating cell types at day 21 of EAM were determined using flow cytometry. D, E) Eosinophilic myocarditis in a 41-year-old female patient with a history of hypereosinophilic syndrome. D) Cardiovascular magnetic resonance (4-Chamber view) shows late gadolinium enhancement (LGE) in the right (blue arrow) and left atrial free wall (yellow arrow) and focal subendocardial LGE of the inferolateral myocardial wall of the left ventricle (red arrow). E) H&E-stained endomyocardial biopsy from the right ventricular septum showing eosinophilic infiltration in the myocardium. A, C) Groups were compared by one-way ANOVA followed by Tukey's multiple comparison test. A) Data shown are from one experiment with 4 mice/group. B) For each population, WT and IL-5Tg were compared by t-test. Data are representative of 2 independent experiments with 5-6 mice/group. C) Data are representative of 2 independent experiments with 3-4 mice/group. Asterisk indicate significance: \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .



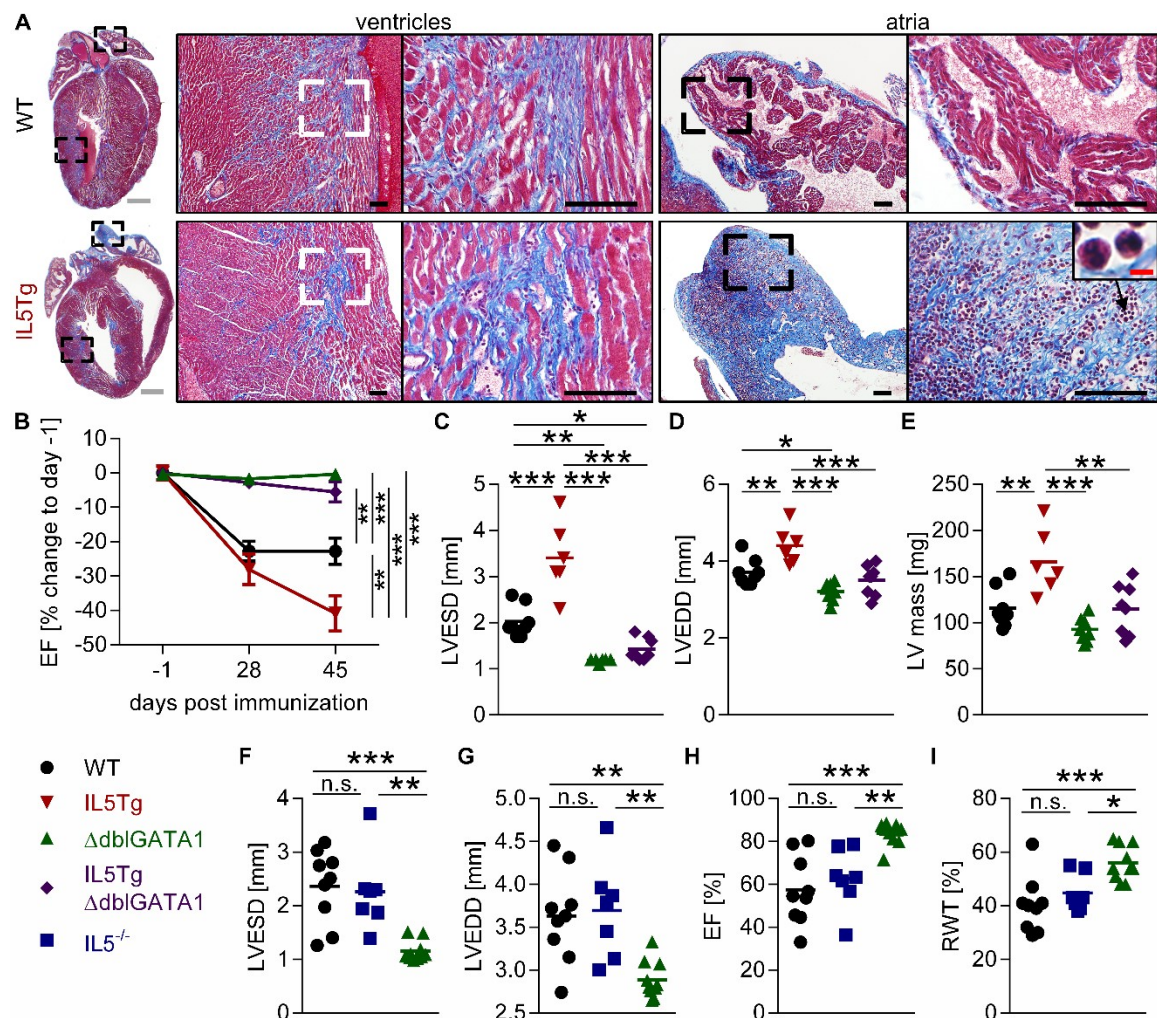


### **Eosinophilic atrial inflammation progresses to severe fibrosis**

By day 45 of EAM, IL-5Tg mice developed ventricular fibrosis similar to WT mice. The severe atrial inflammation in IL-5Tg mice progressed into extensive fibrosis of the atria, often with ongoing eosinophilic inflammation (Figure 23A, Figure 24A-D). We wondered if atrial inflammation/fibrosis was also a common feature of eosinophilic myocarditis in patients. We identified 3 patients with biopsy-confirmed eosinophilic myocarditis and cardiac magnetic resonance imaging seen at the Johns Hopkins Hospital, Department of Cardiology. Two of these three patients showed late gadolinium enhancement (LGE) in the atria, indicating fibrosis or inflammation of the atria. Patient 1 had relatively more atrial than ventricular involvement (Figure 22D, E). Patient 2 had predominantly left ventricular involvement with some LGE of the right ventricle. There was less atrial involvement with patchy LGE located near the annulus. Other studies have also described atrial involvement either alone or together with ventricular inflammation in some cases of eosinophilic myocarditis and giant cell myocarditis<sup>168, 243-247</sup>. This suggests that atrial inflammation and/or fibrosis may also be a feature in some eosinophilic myocarditis patients.

### **Eosinophils drive severe DCMi**

To determine if large numbers of eosinophils could contribute to DCMi severity, we analyzed heart function over time in WT, hypereosinophilic IL-5Tg, and eosinophil-deficient  $\Delta$ dblGATA1 mice. As expected, WT mice developed DCMi and  $\Delta$ dblGATA1 mice showed no change in EF over time. IL-5Tg mice showed an even further reduction in EF compared to WT mice, developing very severe DCMi by day 45 of EAM (Figure 23B). IL-5Tg mice also progressed to more severe dilation of the left ventricle and increased LV mass compared to WT mice (Figure 23C-E). Thus, the level of eosinophilia correlated with DCMi severity, indicating that eosinophils drive progression of myocarditis to DCMi.



**Figure 23: IL-5Tg but not IL-5TgΔdbiGATA1 or IL-5<sup>-/-</sup> mice progress to severe DCMi**

A) Representative images of Masson's trichrome stained heart sections from day 45 of EAM. B) Serial echocardiography was performed on day -1, 28, and 45 of EAM. EF for individual mice was normalized to EF of day -1. Mean  $\pm$  SEM of combined data from two independent experiments with 5-8 mice per group is shown. C-I) Echocardiography was performed on day 45 of EAM in the indicated strains. A, C-I) Data are representative of 2 independent experiments with 5-9 mice/group. B-I) Groups were compared by one-way ANOVA followed by Tukey's multiple comparisons test for all groups against each other. In B) % change in EF on day 45 was compared for all groups. Grey scale bars: 1mm, black scale bars: 100 $\mu$ m, red scale bars: 5 $\mu$ m. Asterisk indicate significance: \* $p$ <0.05, \*\* $p$ <0.01, \*\*\* $p$ <0.001.

### IL-5 is dispensable for progression to DCMi

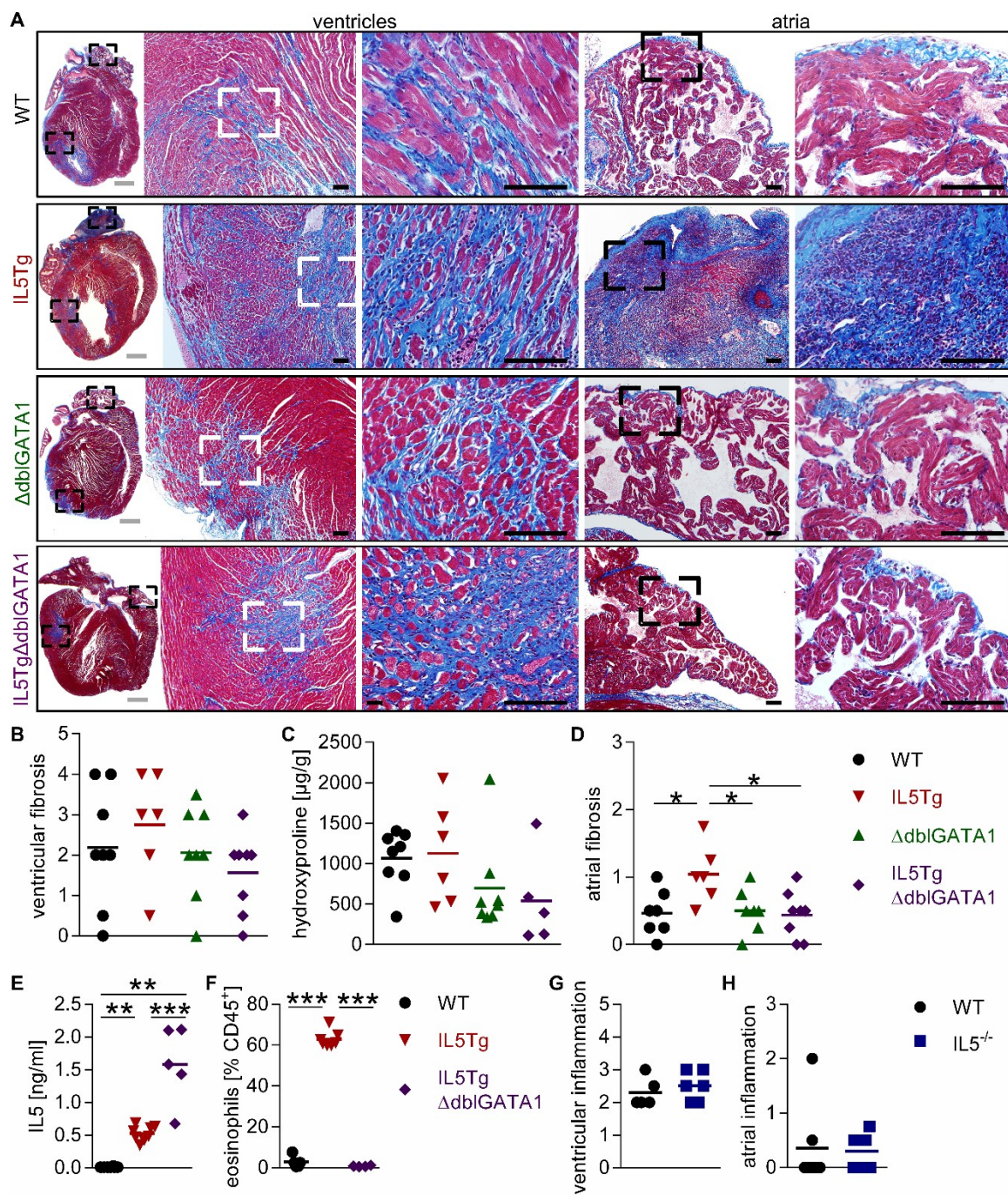
To discriminate between the effects of IL-5 and eosinophils, we crossed IL-5Tg mice with  $\Delta$ dblGATA1 mice. Resulting IL-5Tg $\Delta$ dblGATA1 mice had high serum levels of IL-5 but lacked eosinophils (Figure 24E, F). IL-5Tg $\Delta$ dblGATA1 mice were completely protected from the decrease in EF and LV dilation seen in IL-5Tg mice (Figure 23B-E). Like  $\Delta$ dblGATA1 mice, IL-5Tg $\Delta$ dblGATA1 mice were completely protected from DCMi. This demonstrates that high IL-5 levels were not directly responsible for progression to DCMi, but rather eosinophils were driving this process.

We assessed IL-5<sup>-/-</sup> mice to determine if IL-5 is necessary for the development of DCMi following myocarditis. Myocarditis severity in IL-5<sup>-/-</sup> mice was comparable to WT mice (Figure 24G, H) and IL-5<sup>-/-</sup> mice developed DCMi with the same severity as WT mice, showing no difference in LVEDD, LVESD, EF, or RWT (Figure 23F-I). Together, these data led us to conclude that IL-5 was neither necessary for DCMi development, nor did it drive disease progression. In contrast, eosinophils were required for DCMi development and caused severe DCMi when present in large numbers.

### Figure 24: Eosinophils rather than IL-5 contribute to severe DCMi and atrial inflammation / fibrosis

A-D) Mice were analyzed at day 45 of EAM. A) Heart sections were stained with Masson's trichrome and are shown representatively for all indicated mouse strains. Mice from different experiments are shown. Grey scale bars: 1mm, black scale bars: 100 $\mu$ m. B-D) The extent of fibrosis was determined by scoring fibrotic areas on histological sections (B, D) and by quantifying the amino acid hydroxyproline in heart samples (C). B-D) Data are representative of 2 independent experiments with 5-9 mice/group. E) IL-5 concentration in serum of naïve mice was determined by ELISA. F-H) Mice were analyzed at day 21 of EAM. F) Heart-infiltrating eosinophils were quantified by flow cytometry. G, H) Severity of inflammation was scored on H&E stained heart sections. G) Data are representative of 2 independent experiments with 5-6 mice/group. H) Data are pooled from 2 independent experiments with 2-6 mice/group. Groups were compared by one-way ANOVA followed by Tukey's multiple comparisons test (B-F) or by Mann Whitney test (G, H). Asterisk indicate significance: \*p<0.05, \*\*p<0.01, \*\*\*p<0.001.





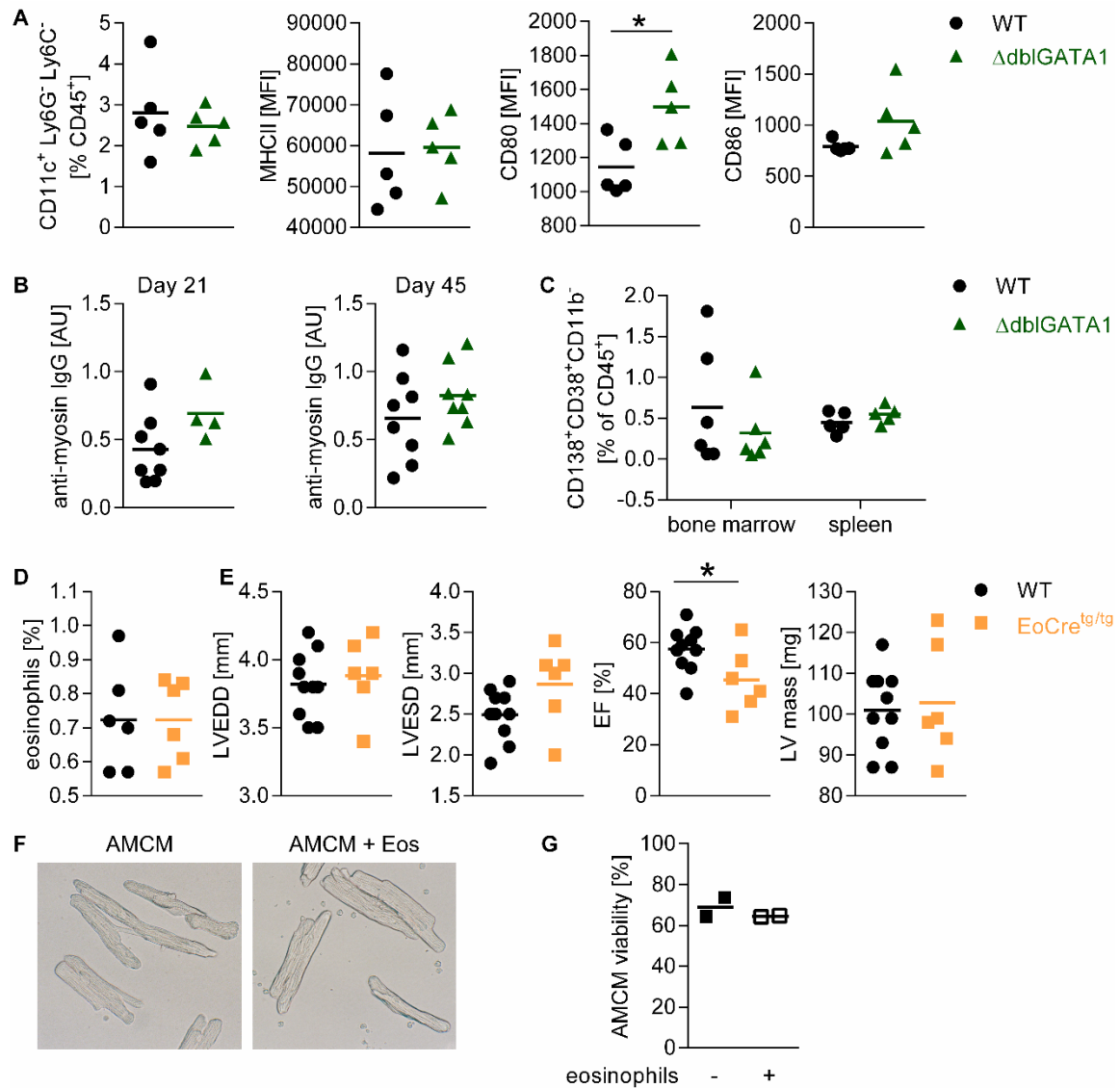
### **Eosinophils do not cause DCMi through effects on dendritic cells, antibody production, or cytotoxic effects**

In allergic asthma, eosinophils are required for dendritic cell (DC) activation and suppress downstream Th17 responses<sup>248</sup>. The frequency of cardiac DCs and expression of costimulatory molecules on their surface was not reduced in  $\Delta$ dblGATA1 compared to WT mice on day 10 of EAM (Figure 25A). We did not observe differences in Th17 cells either (Figure 19, Figure 20). Eosinophils have also been reported to promote plasma cell survival in the bone marrow and IgA production in the intestine<sup>249-251</sup>. While antibodies are neither required nor sufficient for induction of myocarditis in BALB/c mice, it is unclear whether they play a role in progression to DCMi<sup>12, 252, 253</sup> and pathogenic autoantibodies have been demonstrated in other experimental models and in patients<sup>254, 255</sup>.  $\Delta$ dblGATA1 and WT mice had comparable levels of cardiac myosin-specific IgG in serum on days 21 and 45, and of plasma cells on day 21 of EAM (Figure 25B, C). Thus, it is unlikely that eosinophils drive DCMi through effects on antibody production. Previously, eosinophil granule proteins were shown to activate mast cells<sup>45</sup>. We did not observe differences in mast cell numbers (Figure 19) or in serum IgE (not shown) between WT and  $\Delta$ dblGATA1 mice.

Eosinophils are thought to damage the heart by releasing cytotoxic granule proteins<sup>39, 43, 44, 186</sup>. We tested whether the granule protein eosinophil peroxidase, which is pathogenic in a colitis model<sup>256</sup>, was important for DCMi. Mice lacking functional eosinophil peroxidase (homozygous EoCre<sup>tg/tg</sup> mice with recombination into the eosinophil peroxidase locus) were not protected from DCMi (Figure 25D, E). Using *in vitro* co-culture of primary cardiomyocytes from adult WT mice with eosinophils, we found no effect of eosinophils on the survival of cardiomyocytes (Figure 25F, G). Some cardiomyocyte necrosis was evident on the histological sections from all strains of mice during EAM. However, the extent of necrosis was not increased in strains with higher numbers of heart-infiltrating eosinophils (Figure 18A, Figure 21A). We conclude from these data that eosinophils do not drive DCMi through cytotoxic effects on cardiomyocytes.

**Figure 25: Eosinophils do not drive DCMi through effects on dendritic cells, antibody production, cytotoxic effects on cardiomyocytes or eosinophil peroxidase**

A) Dendritic cell frequency and activation in the heart at day 10 of EAM was determined by flow cytometry. B) Myosin-specific IgG was quantified in serum on days 21 and 45 of EAM. C) Plasma cell frequency in the bone marrow of WT and  $\Delta$ dblGATA1 mice on day 21 of EAM. D) Eosinophil frequency in the blood of WT and EoCretg/tg mice on day 45 of EAM was determined by flow cytometry. E) Echocardiography of WT and EoCretg/tg mice on day 45 of EAM. Mice homozygous for the EoCre transgene are functionally eosinophil peroxidase knockouts. F, G) Adult mouse cardiomyocytes (AMCM) from WT mice were cultured in vitro for 20 h with or without eosinophils isolated from the blood of IL-5Tg mice. Dead (round, detached) and viable (adherent, rod-shaped) AMCM were counted under the microscope at 40X magnification. Viability is depicted in (G). Groups were compared by t-test. Asterisk indicate significance: \* $p < 0.05$ .



### Eosinophils in the heart are activated

Eosinophil activation results in upregulation of SiglecF<sup>257</sup>, a surface receptor that triggers eosinophil apoptosis as part of a negative feedback loop<sup>258-260</sup>. Eosinophils in the heart of WT and IL-5Tg mice showed increased expression of SiglecF compared to splenic eosinophils during EAM (Figure 26A). Eosinophils from IL-5Tg mice had higher SiglecF expression than WT mice in both spleen and heart. Cardiac eosinophils also expressed higher levels of CD11b (Figure 26B). This concurs with our previously reported findings that eosinophils in the heart of NK-cell depleted mice are activated during EAM showing increased expression

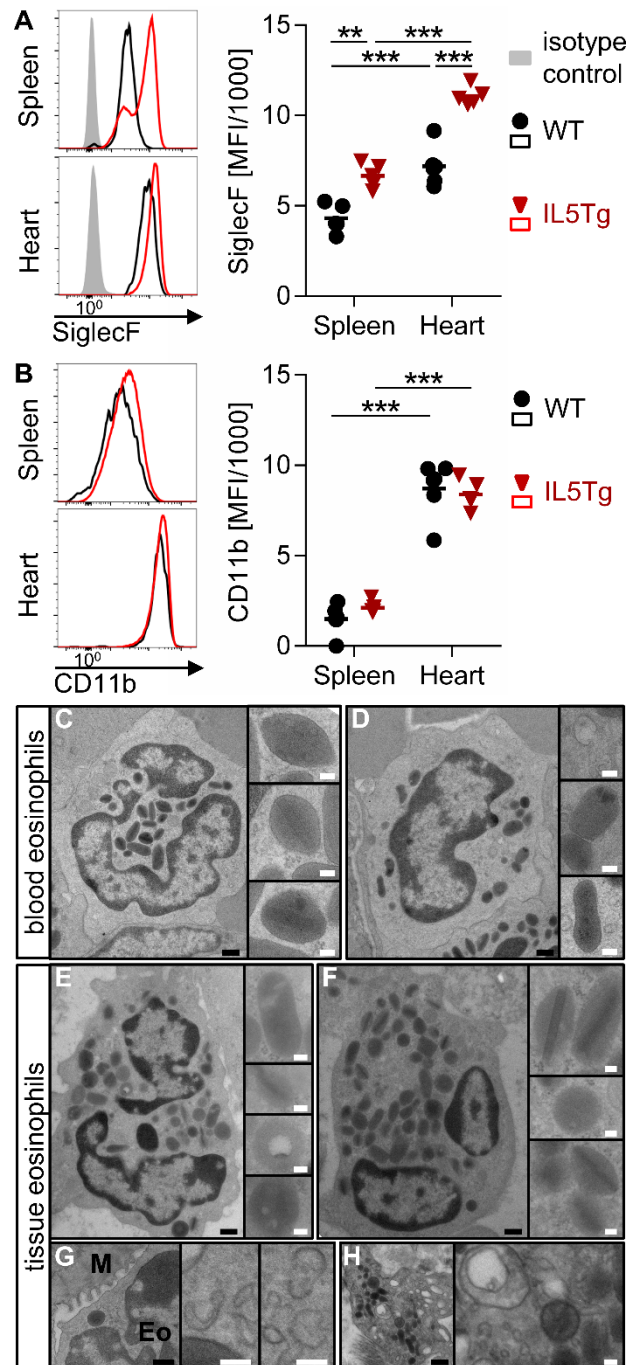


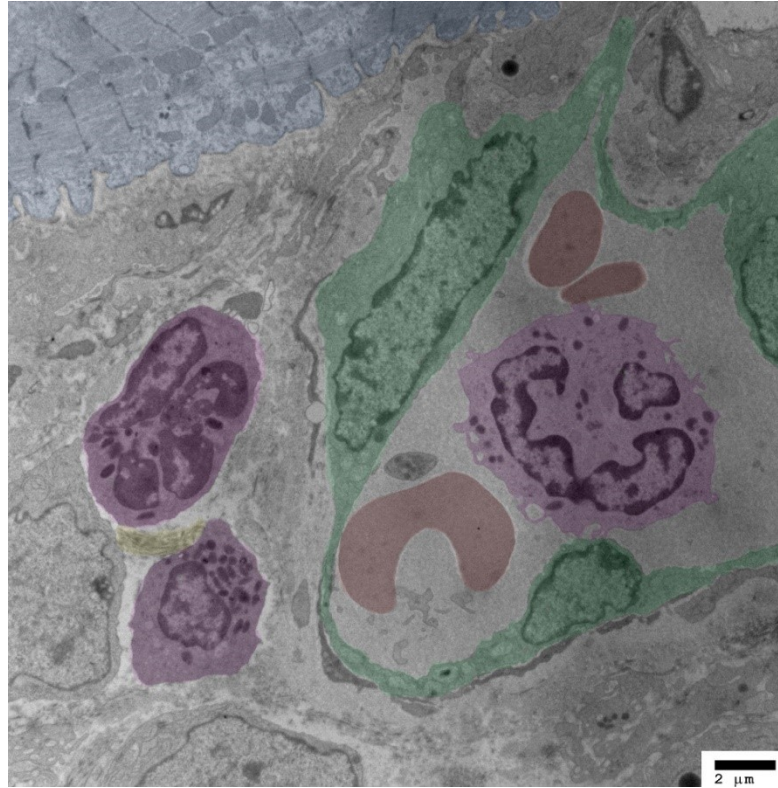
of SiglecF, CD11b, and an altered transcriptional profile as compared to splenic eosinophils from the same mice<sup>187</sup>.

We used electron microscopy to determine the extent of eosinophil degranulation in the heart. The ultrastructure of tissue-infiltrating eosinophils was compared to blood eosinophils on cardiac sections from an IL-5Tg mouse (Figure 27). Blood eosinophils showed largely regular architecture of mature granules with an electron dense core surrounded by an electron-lucent matrix (Figure 26C, D). Granules in tissue eosinophils were also mostly intact without apparent signs of degranulation (Figure 26E, F). The number of granules was comparable in blood and tissue eosinophils (not shown). Only rarely did we observe granules with a loss of matrix or core as described for models of allergic asthma, colitis, or in vitro degranulation<sup>256, 261, 262</sup> (Figure 26E, H). Free extracellular granules or compound exocytosis of granules were not observed. We therefore conclude that eosinophils in the heart do not degranulate on a large scale during EAM. However, vesiculo-tubular organelles and ruffled membranes were very prominent in the cytoplasm of tissue-infiltrating eosinophils (Figure 26G, H), suggesting that eosinophils might be releasing specific granule contents through piecemeal degranulation.

**Figure 26: Heart-infiltrating eosinophils show an activated phenotype.**

A, B) Eosinophils were gated by flow cytometry as viable CD45+Ly6C-Ly6Glo/intSiglecF+ cells on day 21 of EAM. Histograms and mean fluorescent intensity (MFI) for SiglecF (A) and side scatter (SSC, B) are shown. Groups were compared by one-way ANOVA followed by Tukey's multiple comparisons test for all groups against each other. Data are representative of 3 independent experiments with 4-6 mice/group. C-H) Electron microscopy of eosinophils from an IL-5Tg mouse at day 21 of EAM. Representative images of intravascular (blood) eosinophils (C-D) and tissue-infiltrating eosinophils (E-H) are depicted. Small inserts show high magnification of individual granules (C-F) or cytoplasmic vesiculo-tubular structures (G-H). Black scale bars: 500nm; white scale bars: 100nm. Asterisk indicate significance: \*p<0.05, \*\*p<0.01, \*\*\*p<0.001.





**Figure 27: Electron microscopy of heart-infiltrating and blood eosinophils**

Pseudocoloring: cardiomyocytes (blue), endothelial cells (green), red blood cells (red), eosinophils (pink), collagen fibers (yellow).

### **Eosinophils account for the majority of IL-4 expressing cells in EAM**

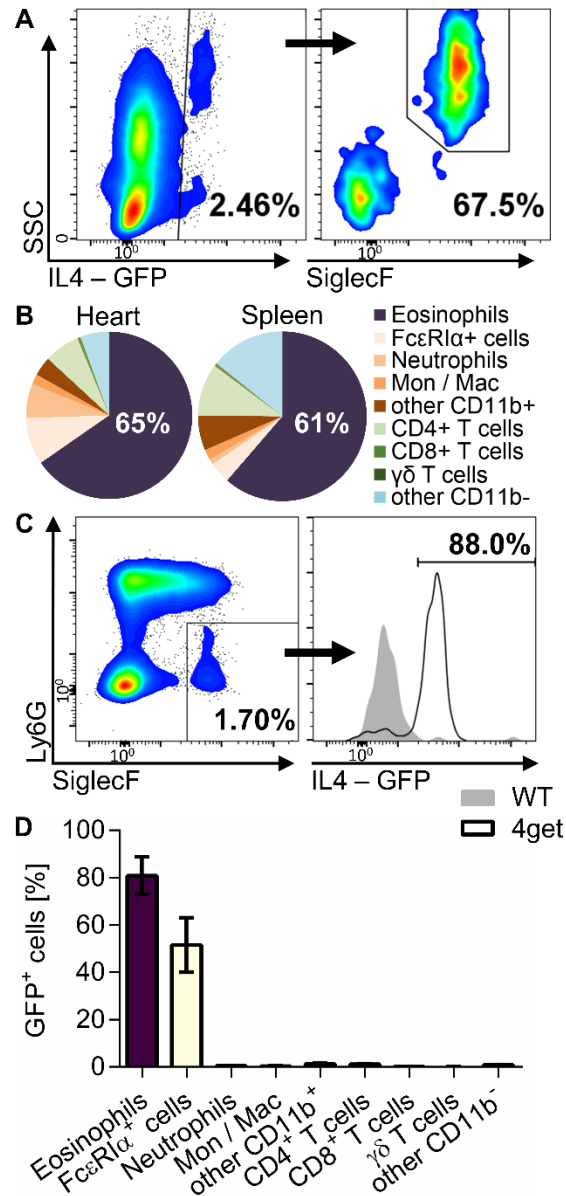
Eosinophil granules harbor numerous cytokines in addition to granule proteins and can selectively release them upon stimulation<sup>99, 263</sup>. Of these, eosinophil-derived IL-4 has been shown to affect several physiological processes<sup>264-267</sup>. To assess IL-4 production by eosinophils in myocarditis, we employed the IL-4 reporter mouse 4get, in which IL-4-expressing cells are GFP<sup>+</sup>. Eosinophils accounted for the majority (over 60%) of IL-4-expressing cells in myocarditis in 4get mice (Figure 28A, B). Over 80% of eosinophils were GFP<sup>+</sup> indicating that the vast majority of eosinophils expressed IL-4 (Figure 28C, D). Hence, eosinophils are the major IL-4-producing cells in myocarditis.

### **IL-4<sup>-/-</sup> mice are protected from DCMi**

Next, we determined the role of IL-4 in myocarditis and DCMi. We previously published that IL-4<sup>-/-</sup> mice develop myocarditis with the same severity as WT mice<sup>239</sup>. When we analyzed heart function in IL-4<sup>-/-</sup> mice by echocardiography, we found that they were completely protected from DCMi. Like  $\Delta$ dbIGATA1 mice, IL-4<sup>-/-</sup> mice had reduced LVESD and LVEDD and preserved EF (Figure 29A). Taken together with the data showing that eosinophils comprise the majority of IL-4-expressing cells, these results strongly suggested that eosinophils drive progression of myocarditis to DCMi through their production of IL-4.

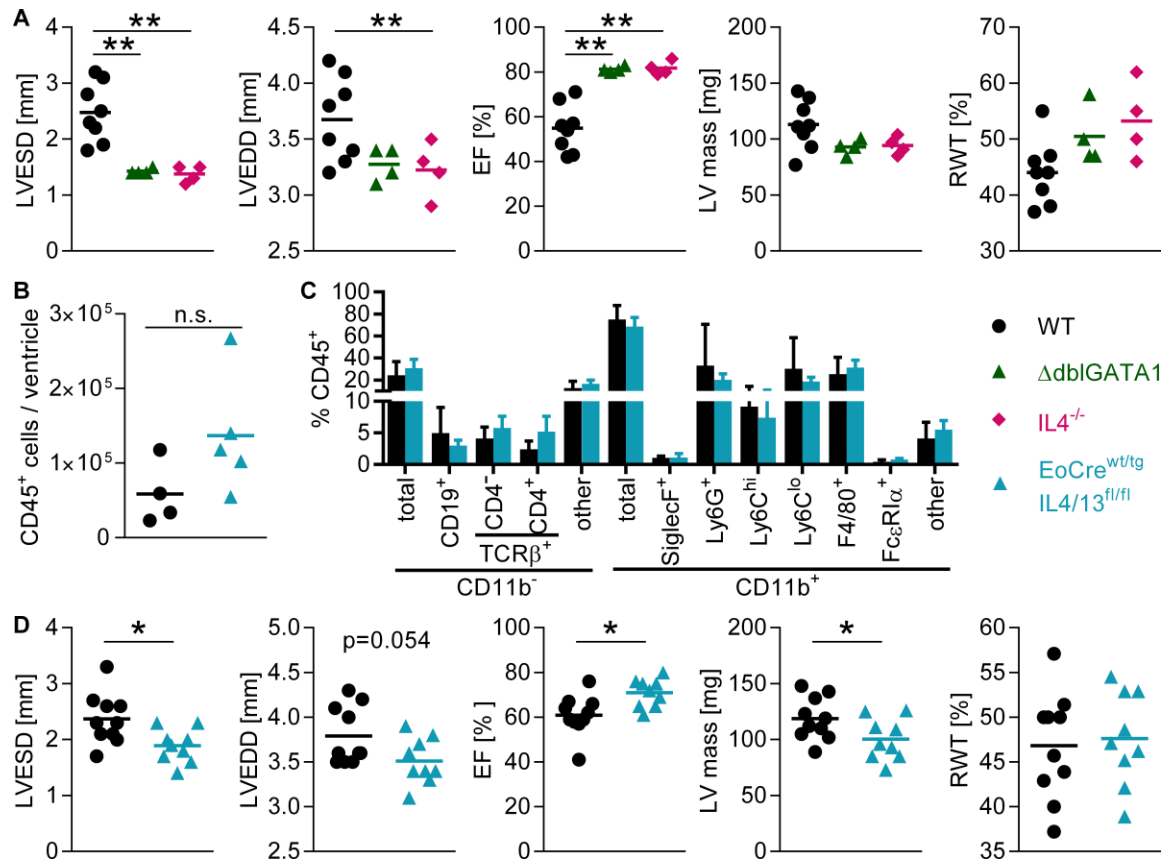
### **Eosinophil-specific deletion of IL-4 ameliorates DCMi**

To assess whether IL-4 derived from eosinophils is necessary for DCMi development, we generated mice with eosinophil-specific IL-4 and IL-13 deletion. We crossed mice with eosinophil-specific Cre recombinase expression (EoCre) with IL-4/13<sup>fl/fl</sup> mice. Resulting EoCre<sup>wt/tg</sup>IL-4/13<sup>fl/fl</sup> mice developed myocarditis similar to WT mice. Absolute numbers and composition of infiltrating CD45<sup>+</sup> cells were comparable (Figure 29B, C). However, at day 45 of EAM, EoCre<sup>wt/tg</sup>IL-4/13<sup>fl/fl</sup> mice showed decreased LVESD, increased EF and decreased LV mass compared to WT controls (Figure 29B). Ventricular and atrial fibrosis was similar in WT, IL-4<sup>-/-</sup>, and EoCre<sup>wt/tg</sup>IL-4/13<sup>fl/fl</sup> mice (Figure 30A-C). These results show that eosinophils drive dilated cardiomyopathy through the production of IL-4. Eosinophil-specific IL-4 deletion resulted in a milder phenotype compared to IL-4<sup>-/-</sup> mice (Figure 29). This is likely a result of incomplete Cre-mediated recombination of the IL-4/13 locus; crossing EoCre mice to the deleter strain ROSA-DTA resulted in a reduction, but not complete ablation, of eosinophils during EAM (Figure 30D). We can not exclude effects from residual IL-4 production by other cell types or other eosinophil-derived mediators. Expression of tissue remodeling-associated genes *Mmp2* and *Timp2*, which was reduced in  $\Delta$ dbIGATA1 mice, was also diminished in EoCre<sup>wt/tg</sup>IL-4/13<sup>fl/fl</sup> mice (Figure 30E), suggesting that these changes in gene expression are mediated through eosinophil-derived IL-4. This demonstrates that eosinophils lead to DCMi through their production of IL-4.



**Figure 28: Eosinophils are the major producers of IL-4 in myocarditis**

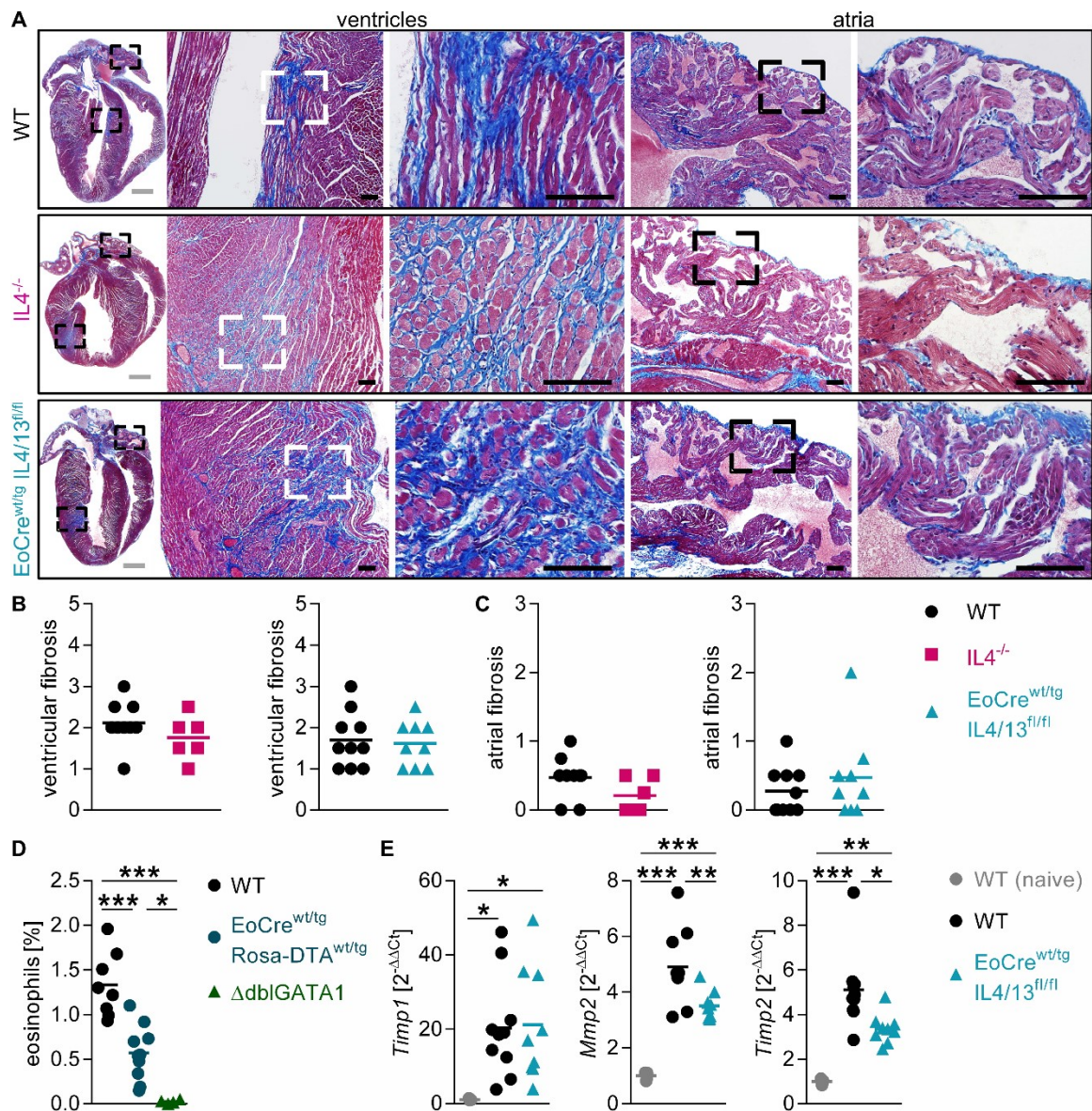
Mice were analyzed at day 21 of EAM. A) Gating of IL-4 expressing (GFP<sup>+</sup>) cells out of viable, CD45<sup>+</sup> cells in the heart of 4get mice (IL-4-GFP reporter mice). B) Frequency of cell types out of heart-infiltrating or splenic GFP<sup>+</sup> cells (average of 3 mice). C) Gating of eosinophils out of viable, CD45<sup>+</sup> cells and GFP expression in eosinophils from WT and 4get mice. D) Frequency of GFP<sup>+</sup> cells among different heart-infiltrating cell types. Data are representative of 2 independent experiments with 3 mice.



**Figure 29: Eosinophil-derived IL-4 promotes DCMi**

A, D) Heart function was assessed by echocardiography on day 45 of EAM in the indicated strains. A) Data are representative of 3 independent experiments with 4-8 mice/group. B, C) Infiltrating cells in the ventricle were quantified by flow cytometry on day 21 of EAM. Data are from one experiment with 4-5 mice/group. D) Data are from one experiment with 9-10 mice/group. Groups were compared by one-way ANOVA followed by Tukey's multiple comparisons test for all groups against each other (A) or by t-test (B-D). Asterisk indicate significance: \* $p < 0.05$ , \*\* $p < 0.01$ .





**Figure 30: EoCre<sup>wt/tg</sup>IL-4/13<sup>fl/fl</sup> mice develop fibrosis to the same extent as WT mice but show altered tissue remodeling similar to ΔdbIGATA1 mice**

Mice were analyzed at day 45 of EAM. A) Heart sections were stained with Masson's trichrome and are shown representatively for all indicated mouse strains. Mice from different experiments are shown. Grey scale bars: 1mm, black scale bars: 100μm. B, C) The extent of fibrosis was determined by scoring fibrotic areas on histological sections. Groups were compared by Mann-Whitney test (no significant differences). D) Eosinophil frequency in blood was determined by flow cytometry. E) Gene expression in heart

homogenates was analyzed by qPCR. D, E) Groups were compared by one-way ANOVA followed by Tukey's multiple comparisons test. Asterisk indicate significance: \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .

## Discussion

In this study, we demonstrated that eosinophils are necessary for the progression of autoimmune myocarditis to DCMi. Mice lacking eosinophils developed myocardial inflammation similar to WT mice following EAM induction but did not develop DCMi and showed no signs of heart failure. Eosinophils accounted for 1-3% of heart-infiltrating CD45<sup>+</sup> cells in WT mice. While their absence did not prevent myocarditis or affect its severity, it had a prominent effect on DCMi development. This shows that the severity of myocarditis does not necessarily determine the long-term disease outcome, but specific infiltrating cell types are decisive for disease progression.

IL-5Tg mice can develop spontaneous cardiac enlargement and eosinophil infiltration at an unknown rate<sup>195</sup>. EAM induction results in myocarditis in nearly all mice, making it a much more reliable model and more feasible to study because of the defined onset. EAM in IL-5Tg mice is a model of eosinophilic myocarditis with hypereosinophilia. HES patients frequently develop endomyocardial thrombi which are thought to result in endomyocarditis and endomyocardial fibrosis<sup>33, 182</sup>. We rarely observed intra-atrial or intraventricular thrombi and no signs of endocarditis in IL-5Tg mice following EAM induction. HES patients can also develop eosinophilic myocarditis or pericarditis<sup>33, 182</sup>. In IL-5Tg mice, inflammation was primarily located in the ventricular myocard and atria; pericarditis was mild. Most notably, IL-5Tg mice develop extensive eosinophil infiltration of the myocard associated with a loss in EF, which is also observed in HES patients.

We previously reported on acutely fatal eosinophilic myocarditis developing in IFN $\gamma$ <sup>-/-</sup>IL-17A<sup>-/-</sup> mice<sup>189</sup>. Induction of EAM in IL-5Tg mice offers a second model of eosinophilic myocarditis with several differences: 1) IL-5Tg mice have hypereosinophilia, while IFN $\gamma$ <sup>-/-</sup>IL-17A<sup>-/-</sup> mice have normal peripheral blood eosinophil



levels. 2) Eosinophils reach over 60% of heart-infiltrating cells in IL-5Tg mice, and about 30% in IFN $\gamma$ <sup>-/-</sup>IL-17A<sup>-/-</sup> mice. 3) Eosinophil migration to the heart depends on the eotaxin-CCR3 pathway in IFN $\gamma$ <sup>-/-</sup>IL-17A<sup>-/-</sup> mice<sup>74</sup>. For IL-5Tg mice, the mediators of eosinophil trafficking are not known. Eotaxin expression is comparable to WT mice in the ventricles and slightly increased in the atria. 4) The severity of myocardial inflammation is moderate in IL-5Tg mice while IFN $\gamma$ <sup>-/-</sup>IL-17A<sup>-/-</sup> mice develop massive inflammation that encompasses almost the entire myocardium and likely causes fatality in these mice. IL-5Tg mice rarely died after EAM induction. Severe atrial inflammation is present in both strains. Importantly, both strains develop heart failure that is rescued by ablation of eosinophils. This confirms a crucial role for eosinophils in driving DCMi.

Severe atrial inflammation and fibrosis were unique features in IL-5Tg mice. Severe atrial fibrosis may have caused atrial fibrillation or arrhythmias in IL-5Tg mice. We noted atrial involvement in 2/3 eosinophilic myocarditis patients described here. A recent case series found that 8 of 9 patients with eosinophilic myocarditis had enlargement of the atria<sup>243</sup>. Another study characterized 6 patients with isolated atrial giant cell myocarditis showing atrial fibrillation, atrial wall thickening, atrial enlargement, and marked atrial inflammation<sup>168</sup>. Similar observations were made in case reports of giant cell myocarditis<sup>244</sup> and eosinophilic myocarditis<sup>34, 245, 246</sup>. Eosinophilic myocarditis has also been identified as a cause of atrioventricular block in one patient<sup>247</sup>. This suggests that atrial inflammation and/or fibrosis are also a feature of some eosinophilic myocarditis patients. Future studies should explore what determines atrial involvement in eosinophilic myocarditis and assess possible pro-arrhythmogenic effects of such inflammation.

The exacerbated heart failure in IL-5Tg mice was due to the high numbers of eosinophils. IL-5 was not directly responsible for this phenotype because IL-5Tg $\Delta$ dblGATA1 with high serum IL-5 but no eosinophils were protected from DCMi and IL-5<sup>-/-</sup> mice exhibited DCMi comparable to WT mice. This clearly demonstrated that the role of IL-5 was limited to promoting eosinophilia. Eosinophils, in turn, were responsible for the DCMi phenotype and increasing numbers resulted in more severe DCMi. As a result of

the size difference between atria and ventricles, most cardiac eosinophils located to the ventricles despite more severe inflammation in the atria. It is unclear whether eosinophil infiltration in the atria or ventricles were responsible for the severe DCMi observed in these mice. Because of the rarity of eosinophilic myocarditis, it is not known what percentage of eosinophilic myocarditis patients progress to DCMi. In a study on the survival of 112 biopsy-proven myocarditis patients, 4 out of 7 patients with eosinophilic myocarditis underwent cardiac transplantation within 1 year of diagnosis<sup>113</sup>, suggesting that transplant-free survival may be particularly poor among these patients. In one series of eosinophilic myocarditis patients, EF was impaired in all 5 patients and severely impaired in 2 patients<sup>268</sup>. More studies will be necessary to determine if eosinophilic myocarditis is associated with progression to DCMi in patients.

In previous studies, we showed that IL-17A is another driver of DCMi<sup>197, 240</sup>. In the absence of IL-17A, mice are protected from DCMi and show markedly reduced levels of cardiac fibrosis. Eosinophils drive DCMi through a distinct mechanism. Lack of eosinophils or eosinophil-derived IL-4 did not affect fibrosis or collagen levels in the heart but rather affected expression of tissue remodeling-associated genes. In this paper, we demonstrate that mice can develop cardiac fibrosis but still retain a high ejection fraction. Therefore, the level of fibrosis is not a necessary negative regulator of cardiac function. It is possible that both increased collagen production induced by IL-17A and tissue remodeling by matrix metalloproteinases stimulated by eosinophils are necessary to drive progression of myocarditis to DCMi. Another conceivable explanation would be a direct link between Th<sub>17</sub> responses and eosinophils as it was suggested in recent studies. The Th<sub>17</sub> effector cytokine GM-CSF enhances eosinophilopoiesis, induces cytokine secretion, and promotes survival<sup>269, 270</sup>.

We tested numerous hypotheses regarding the mechanism by which eosinophils promote DCMi: direct cytotoxic effects, activation of other immune cell types (T cells or dendritic cells), effects on antibody production, and eosinophil peroxidase-driven effects. None of these were supported by the data. However, eosinophils in the heart showed an activated phenotype with formation of vesiculo-tubular structures in

the cytoplasm without a large extent of degranulation. We hypothesized that eosinophils may be releasing specific mediators to promote DCMi.

We showed that eosinophil-derived IL-4 is critical for progression of myocarditis to DCMi. Several previous studies have uncovered a role for eosinophils and IL-4 in physiological processes including tissue regeneration, differentiation of beige fat, and glucose homeostasis<sup>264-267</sup>. Here, we report a pathogenic role for eosinophil-derived IL-4. By specifically deleting IL-4 in eosinophils, we demonstrate that eosinophils are indeed the source of IL-4 required for DCMi development. This does not exclude the possibility of additional eosinophil-derived mediators contributing to heart failure.

While eosinophils are not the only cells known to produce IL-4, other cell types are much less likely to be the main source of IL-4 in the heart. Basophils can produce IL-4<sup>271, 272</sup> and can be affected by the  $\Delta$ dblGATA1 mutation that prevents eosinophil development<sup>273</sup>. However, basophils comprised less than 2% of all IL-4-producing cells in myocarditis and there was no difference in the frequency of heart-infiltrating basophils between WT and  $\Delta$ dblGATA1 mice. While T cells can also produce IL-4, they represented less than 10% of all IL-4 expressing heart-infiltrating cells during myocarditis.

Our studies also provide insight into the opposing effects of IL-4 and IL-13 in myocarditis. We previously published that IL-13 is protective in myocarditis and DCMi<sup>239</sup>. IL-13-deficient mice develop severe myocarditis and rapidly progress to heart failure. In contrast, IL-4-deficient mice exhibit myocarditis of similar severity as WT mice<sup>239</sup> but were completely protected from DCMi. While both are hallmark cytokines of a type 2 immune response, the sources of these cytokines are different. IL-4 is mostly expressed by eosinophils and to a lesser extent by CD4<sup>+</sup> T cells. The major source of IL-13 is likely lymphoid: T cells and innate lymphoid cells. IL-13 protects against myocarditis through multiple effects on monocytes and macrophages<sup>239</sup>. IL-4 may act on other target cells. Given these different roles of IL-4 and IL-13 in myocarditis, it is unlikely that the absence of IL-13 in EoCre<sup>wt/tg</sup>IL-4/13<sup>fl/fl</sup> mice is responsible for the observed protective effect.

Currently, no drugs are available to arrest or delay development of DCMi, or to improve long-term survival of DCMi patients. Our results uncovered a new pathway critically involved in the progression of myocarditis to DCMi. Preventing eosinophilia or blocking IL-4 in patients with myocarditis may halt disease progression and thereby avert the need for heart transplants.

## Chapter 5: Eosinophils in autoimmune diseases

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This chapter is under review at *Frontiers in Immunology* as a review article:

Diny NL, Rose NR, Čiháková D. Eosinophils in autoimmune diseases.

### Summary

Eosinophils are multifunctional granulocytes that contribute to initiation and modulation of inflammation. Their role in asthma and parasitic infections has long been recognized. Growing evidence now reveals a role for eosinophils in autoimmune diseases. In this review, we summarize the function of eosinophils in inflammatory bowel diseases, neuromyelitis optica, bullous pemphigoid, autoimmune myocarditis, primary biliary cirrhosis, eosinophilic granulomatosis with polyangiitis, and other autoimmune diseases. Clinical studies, eosinophil-targeted therapies and experimental models have contributed to our understanding of the regulation and function of eosinophils in these diseases. By examining the role of eosinophils in autoimmune diseases of different organs we can identify common pathogenic mechanisms. These include degranulation of cytotoxic granule proteins, induction of antibody-dependent cell-mediated cytotoxicity, release of proteases degrading extracellular matrix, immune modulation through cytokines, antigen presentation, and pro-thrombotic functions. The association of eosinophilic diseases with autoimmune diseases is also examined, showing a possible increase in autoimmune diseases in patients with eosinophilic esophagitis, hypereosinophilic syndrome and non-allergic asthma. Finally, we summarize key future research needs.

### Introduction

Activation of innate immune cells by pathogen-associated molecular patterns and antigen presentation by dendritic cells can result in priming of autoreactive T and B cells and set off an adaptive immune response against self-antigens<sup>274-276</sup>. Possible roles for innate immune cells exist not only in the initiation stage of

autoimmune diseases, but also in the modulation and propagation of inflammation and tissue destruction. Such roles have been proposed for neutrophils<sup>277</sup>, natural killer cells<sup>278, 279</sup>, macrophages<sup>280</sup>, dendritic cells<sup>281, 282</sup>, innate lymphoid cells<sup>283</sup>, and mast cells<sup>284</sup>. Eosinophils have been recognized as part of the inflammatory infiltrate in several organ-specific autoimmune diseases, but their potential role in autoimmune diseases has not been addressed comprehensively.

The aim of this review is to synthesize the role of eosinophils in different autoimmune diseases and explore potential unifying effector mechanisms. We also address the association of autoimmune diseases with eosinophil-associated disease like asthma and eosinophilic esophagitis.

### **Possible eosinophil effector functions in autoimmune diseases**

Eosinophils are extremely versatile effector cells that damage tissues or modulate the activity of other immune and stromal cells (see Chapter 1). One could envision many of these effector functions playing a role in the context of autoimmune diseases as well (Figure 31). Damage of tissue and cells is a feature of many organ-specific autoimmune diseases. Eosinophils are well known for their strong cytotoxic properties, mediated mostly through granule proteins. This could contribute to organ destruction in autoimmune inflammation.

The ability of eosinophils to bind antibodies and consequently degranulate and kill cells links the adaptive autoimmune response to eosinophil effector functions. Eosinophils express complement receptors<sup>285</sup> and Fc-receptors (Fc $\alpha$ R, Fc $\gamma$ RI-III, Fc $\epsilon$ RI-II) either constitutively or under inflammatory conditions<sup>286-289</sup>. As a result, they are capable of antibody-dependent cellular cytotoxicity to parasites and mammalian targets<sup>290-292</sup>. In autoimmune diseases, eosinophils may kill host cells bound by autoantibodies.

Eosinophils also interact with stromal cells. Actively degranulating eosinophils are frequently found in areas of fibrogenesis, suggesting a potential profibrotic role<sup>44, 293-295</sup>. Granule proteins and eosinophil-derived TGF $\beta$ 1 were demonstrated to affect tissue remodeling and fibrosis. Eosinophils can promote fibroblast

proliferation<sup>296-298</sup>, proteoglycan accumulation<sup>299</sup>, matrix metalloproteinase and TGF $\beta$  expression, and extracellular matrix protein synthesis<sup>300</sup>. These profibrotic functions of eosinophils may add to tissue dysfunction in autoimmune diseases.

In chronic inflammatory conditions, eosinophils preferentially locate to nerves<sup>211, 301-303</sup>. This interaction results in activation of eosinophils<sup>304-306</sup>, nerve damage<sup>307, 308</sup>, altered nerve growth<sup>309, 310</sup>, neuropeptide release<sup>311</sup>. Contact between eosinophils and nerves has functional consequences. For example, it is a cause of airway hypersensitivity in asthma<sup>88, 312</sup>.

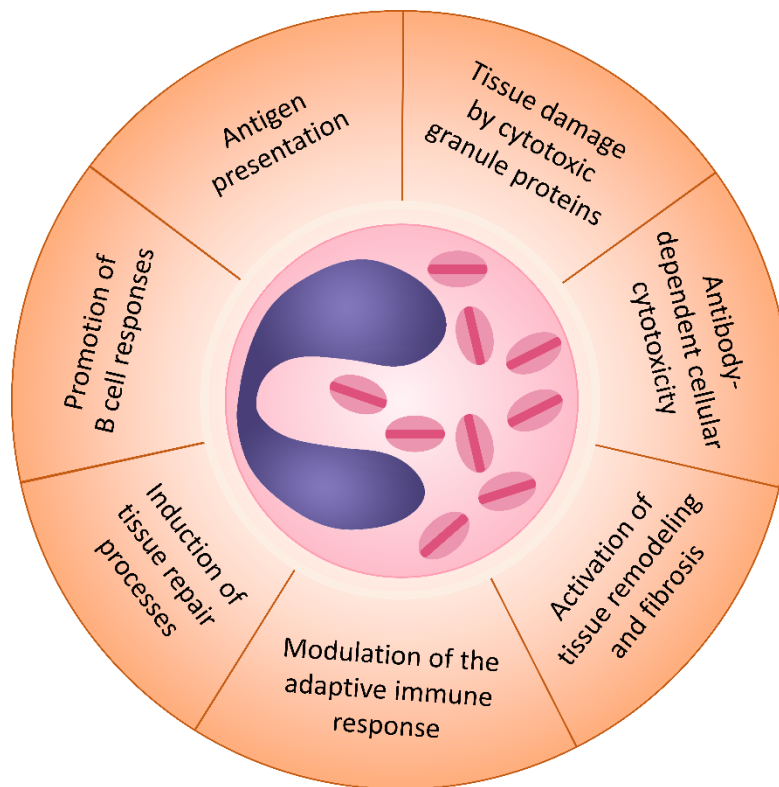
Eosinophils can form extracellular DNA traps by quickly releasing mitochondrial DNA and granule proteins<sup>313</sup>. These structures bind and kill pathogens and contribute to tissue injury in inflammatory conditions<sup>314</sup>. DNA extracellular traps have been described in allergic asthma<sup>315</sup>, drug hypersensitivity reactions, and allergic contact dermatitis<sup>316</sup>. Eosinophils may initiate or perpetuate inflammation by releasing cytokines and chemokines and by interacting with other innate immune cells. For example, eosinophils release MBP, IL-9, stem cell factor, or nerve growth factor, which affect mast cell maturation, survival, and histamine release<sup>317-320</sup>.

Eosinophils can also influence the adaptive immune response. They are capable antigen presenting cells that upregulate MHCII and co-stimulatory molecules in the context of parasitic infection or allergic asthma<sup>321-323</sup>. Moreover, eosinophils migrate to draining lymph nodes<sup>324</sup> and *in vitro* experiments have demonstrated their ability to present antigen to and activate T cells<sup>325-327</sup>. Through antigen presentation, eosinophils may be involved in the initiation of autoimmune responses.

Eosinophil granules contain numerous cytokines such as IL-4, IL-13, IL-25, TGF $\beta$ , IL-10, or IDO<sup>81</sup>, which suggests an ability to affect T cell differentiation. Eosinophils were shown to suppress Th1/Th17 differentiation<sup>248</sup> or activate Th2 responses in draining lymph nodes<sup>328</sup>. In addition, they modulate dendritic cell activity, thereby indirectly affecting T cell differentiation<sup>329</sup>. Eosinophils also shape the humoral immune

response. In the bone marrow, eosinophils stimulate plasma cell survival by producing IL-6 and APRIL<sup>251</sup> and in the intestine, they promote class-switching to IgA<sup>249, 250</sup>. These properties enable eosinophils to shape the adaptive immune response in autoimmune diseases.

Eosinophils may also fulfill immune regulatory and protective functions. Eosinophil-derived mediators like TGF $\beta$  and TGF $\alpha$ <sup>330</sup>, platelet derived growth factor<sup>331</sup>, vascular endothelial growth factor<sup>332</sup>, and fibroblast growth factor<sup>333</sup> can all contribute to tissue repair and angiogenesis but can also promote maladaptive fibrosis in chronic autoimmune inflammation. IL-4 released from eosinophils was shown to play a role in liver<sup>265</sup> and muscle<sup>267</sup> regeneration. Whether eosinophils contribute to tissue repair or tissue damage is likely context and disease dependent.



**Figure 31: Possible eosinophil effector mechanisms in autoimmune diseases**



## Role of eosinophils in autoimmune diseases

### Bullous pemphigoid

Bullous pemphigoid is a blistering disease of the skin with a well-established autoimmune etiology<sup>334</sup>. Autoantibodies bind to hemidesmosomal proteins BP180 and BP230 at the dermal-epidermal junction and other extracellular matrix proteins<sup>335-340</sup>. Hemidesmosomes are part of the complexes that anchor the cytoskeleton of basal keratinocytes to the dermis<sup>341</sup>. Autoantibody binding triggers complement activation, recruitment of immune cells, and release of proteases. This results in tissue damage and blistering<sup>342-345</sup>. Together with mast cells and neutrophils, eosinophils infiltrate the dermal-epidermal junction and are thought to play a key role in bullous pemphigoid<sup>346-348</sup>. Increased numbers of peripheral blood eosinophils has long been recognized as a characteristic of bullous pemphigoid patients<sup>349, 350</sup>. A positive correlation between blood eosinophil numbers and disease severity has been observed in some reports<sup>351, 352</sup> but not others<sup>353</sup>. Eosinophilia in bullous pemphigoid patients is likely caused by increased levels of IL-5, which can be detected at high levels in the serum and blister fluid<sup>354, 355</sup>. Keratinocytes in the blisters express eotaxin-1, which directs eosinophil infiltration<sup>354, 356</sup>. Eotaxin-1 expression is positively correlated with the number of infiltrating eosinophils in blisters. Eosinophil localization to the basement membrane zone is autoantibody- and complement-dependent in a human cryosection model of bullous pemphigoid<sup>348</sup>. Eosinophils from blisters release IL-6, IL-8, and IL-1 $\beta$  and show an activated phenotype with high CD11b expression<sup>357</sup>. Blister eosinophils also underwent apoptosis more readily compared to eosinophils from healthy donors<sup>357</sup>.

Several mechanisms by which eosinophils (and other granulocytes) contribute to lesion formation have been identified. Eosinophils in lesional skin were shown to degranulate and granule proteins are deposited in blisters<sup>346, 358</sup>. The eosinophil granule protein ECP can readily be detected in serum and blister fluid of bullous pemphigoid patients<sup>353</sup>. However, it is not clear if granule proteins contribute to tissue damage. Eosinophils and neutrophils have been shown to release proteases, matrix metalloproteinase 9 (MMP9)

and neutrophil elastase, in lesional biopsies and blister fluid. These proteases can degrade extracellular matrix proteins and BP180, which contributes to dermal-epidermal separation and blister formation<sup>359-361</sup>. Blister formation also depends on autoantibodies<sup>362</sup>, which are of the IgG1, IgG4, and IgE subtype<sup>363, 364</sup>. Recently, eosinophils from bullous pemphigoid patients were shown to express the high affinity IgE receptor FcεRIα<sup>365</sup>, which may trigger eosinophil activation by IgE autoantibodies. Additional evidence for a pathogenic role for eosinophils comes from a case report of a patient with hypereosinophilic syndrome and bullous pemphigoid who was treated with imatinib (a tyrosine kinase inhibitor). In response to imatinib both conditions resolved and his eosinophil count normalized<sup>366</sup>.

Taken together, there is strong evidence from patient studies, in vitro experiments, and animal models for a pathogenic role of eosinophils in bullous pemphigoid. In addition to the mouse model of passive antibody transfer, which reproduces blister formation but not eosinophil infiltration<sup>367</sup>, a new model with genetically modified mice has been established<sup>368</sup>. Mice with a deletion in the BP180 (Collagen XVII) gene spontaneously develop eosinophilia, blister formation, itch and eosinophil infiltration into the skin lesions. This new model could be used to test for the requirement and pathologic role of eosinophils (and eosinophil products) in future studies. Novel eosinophil-specific drugs may also help to clarify the role of eosinophils in bullous pemphigoid. Trials of bertilimumab, an anti-eotaxin-1 antibody, and mepolizumab, an anti-IL-5 antibody, are currently ongoing (ClinicalTrials.gov identifiers: NCT02226146, NCT01705795).

### **Inflammatory Bowel diseases**

The etiology of the inflammatory bowel diseases Crohn's disease and ulcerative colitis is not fully understood. Evidence for the involvement of autoimmune processes exists for both<sup>369, 370</sup>. Both diseases are associated with other autoimmune diseases, characterized by lymphocytic infiltration, and respond to corticosteroid treatment. Neither of the diseases are associated with HLA haplotypes. Patients with ulcerative colitis carry autoantibodies against colonic epithelial cells and often perinuclear anti-neutrophilic cytoplasmic antibodies. Specific autoantibodies have not been found in Crohn's disease patients. The

evidence for autoimmunity is stronger in ulcerative colitis than in Crohn's disease. Here, we will discuss the role of eosinophils in both diseases with a focus on ulcerative colitis.

Eosinophils have long been recognized as a prominent feature of the infiltrate in inflammatory bowel diseases<sup>371-375</sup>. Eosinophil numbers in the colon are substantially increased in inflammatory bowel disease patients and display an activated phenotype<sup>375-378</sup>. The number of infiltrating eosinophils is positively correlated with disease severity in ulcerative colitis and Crohn's disease<sup>73, 379-382</sup>. In mouse models, the absence of eosinophils dramatically reduces disease severity. In the model of DSS-induced colitis, two different strains of eosinophil-deficient mice were protected compared to controls<sup>73, 383</sup>. Depletion of eosinophils in a model of colitis due to *Helicobacter hepaticus* infection also reduced disease severity<sup>269</sup>. Similarly, in a model of TNBS-induced colitis, eosinophil-deficient mice fared better, while hypereosinophilic mice developed more severe disease<sup>384</sup>.

Eosinophil migration into the colon mucosa occurs in response to eotaxins. Patients with inflammatory bowel diseases have elevated serum eotaxin-1 levels<sup>379, 385, 386</sup>, which correlates positively with disease activity<sup>379, 385</sup>. Tissue expression of eotaxin-1, and to a lesser extent eotaxin-2, is increased in ulcerative colitis patients and positively correlated with the number of infiltrating eosinophils and histopathologic disease severity<sup>73, 379</sup>. Another study found increased expression of all 3 eotaxins and IL-5 in ulcerative colitis, but only eotaxin-1 correlated with eosinophil numbers<sup>387</sup>. The relative significance of eotaxin-2 and -3 is less clear. Eotaxin-3 was found to be increased in active lesions in ulcerative colitis and to a lesser extent in Crohn's disease<sup>204</sup>. Gene polymorphisms in eotaxin-2 are associated with ulcerative colitis<sup>388</sup>. This suggests that all eotaxins may contribute to eosinophil trafficking. The cellular source of eotaxin-1 was identified as CD68<sup>+</sup> macrophages and epithelial cells<sup>73</sup> or as CD14<sup>+</sup> mononuclear cells<sup>387</sup>. Colonic myofibroblasts express eotaxin-3, which is increased in response to IL-4 and IL-13<sup>204</sup>. In the mouse model of DSS-induced colitis, eotaxin-1 and -2 expression in the colon is increased and deficiency in eotaxin-1, but not in eotaxin-2, decreases eosinophil infiltration. This demonstrates that eotaxin-1 is the major

chemoattractant for eosinophils in experimental colitis<sup>73, 256</sup>. In this mouse model, macrophages are the major eotaxin-1 producing cell type<sup>73, 389</sup>. The increased expression of eotaxins, particularly eotaxin-1, in inflammatory bowel diseases shows that eosinophils are specifically recruited to the site of inflammation.

Electron microscopy and immunohistochemistry of colonic biopsies show degranulation of eosinophils<sup>378, 390, 391</sup>. Eosinophil granule proteins are also found in the feces<sup>377, 392</sup>, gut perfusates<sup>393</sup>, and gut lavage fluids<sup>394</sup> from patients with ulcerative colitis and Crohn's disease. Eosinophil granule proteins in serum or intestine are positively correlated with disease activity in ulcerative colitis<sup>73, 377, 382, 395, 396</sup>. Polymorphisms in the genes of ECP and EPX are associated with inflammatory bowel diseases<sup>397</sup>. These findings suggest a pathogenic role of eosinophil granule proteins in inflammatory bowel diseases. In one study, however, eosinophil activation was observed during the remission phase<sup>398</sup>. EPX is pathogenic in mouse models of DSS- and *Helicobacter hepaticus*-induced colitis. Genetic deficiency or inhibition of EPX reduced disease severity<sup>256, 269</sup>.

Several pathogenic functions of eosinophils have been suggested in recent years. Eosinophils were found to increase mucosal barrier permeability in ulcerative colitis by releasing MBP<sup>399</sup> or corticotropin-releasing factor<sup>400</sup>. IL-22 is increased in patients with ulcerative colitis or Crohn's disease<sup>401, 402</sup> and animal studies showed that it is crucial to restore epithelial homeostasis<sup>403</sup>. IL-22 induces antimicrobial peptides, mucus production and epithelial tight junctions<sup>404</sup>. Recently, eosinophils were identified as the main source of IL-22 binding protein, inhibiting the protective actions of IL-22 in DSS-induced experimental colitis and in patients with inflammatory bowel disease<sup>405</sup>. In another study, eosinophils were found to localize to nerves in the colonic mucosa in ulcerative colitis and Crohn's disease<sup>211</sup>. Th17 responses have been implicated in inflammatory bowel diseases<sup>406</sup>. A possible link between the downstream effector of Th17 responses, GM-CSF, and eosinophils was found recently. GM-CSF enhances eosinophilopoiesis, induces cytokine secretion from eosinophils, and promotes eosinophil survival<sup>269, 270</sup>.

In summary, tissue eosinophils are increased in patients with inflammatory bowel diseases, are associated with disease severity, and are specifically recruited through eotaxin-1. Eosinophils likely contribute to the disease process by releasing granule proteins (EPX) or other mediators that affect the intestinal barrier. Thus, there is strong evidence for a pathogenic role of eosinophils in inflammatory bowel diseases, particularly in ulcerative colitis.

### **Eosinophilic granulomatosis with polyangiitis**

Eosinophilic granulomatosis with polyangiitis (EGPA) was first described by Churg and Strauss in 1951<sup>407</sup>. The disease progresses through 3 overlapping phases: adult-onset asthma, peripheral and tissue eosinophilia, and necrotizing vasculitis with tissue infiltration of eosinophils<sup>163, 408, 409</sup>. EGPA is an idiopathic type of small vessel vasculitis and is also part of the hypereosinophilic syndromes<sup>410</sup>. It is associated with HLA and IL-10 polymorphisms<sup>411</sup>. About 40% of EGPA patients have perinuclear anti-neutrophilic cytoplasmic (ANCA) antibodies against myeloperoxidase (MPO), resulting in the classification of EGPA as an ANCA-associated vasculitis<sup>163, 409</sup>. The presence or absence of ANCA in EGPA may indicate 2 clinical subtypes with different organ involvement. ANCA-positive patients have more frequent vasculitis and glomerulonephritis, whereas ANCA-negative patients have more frequent heart and lung involvement<sup>32, 35</sup>. Blood and tissue eosinophilia are diagnostic criteria for EGPA, yet little is known about the pathogenic role of eosinophils in this disease<sup>409</sup>. One reason for the absence of mechanistic data is the lack of suitable animal models. The transfer of MPO-positive human serum to mice causes vasculitis, but the eosinophilic component is missing<sup>412</sup>. Therefore, all knowledge about the role of eosinophils in EGPA comes from patient studies. An increased eosinophil count during active disease is associated with increased Th2 cytokines IL-5 in serum<sup>413, 414</sup> and increased production of IL-4, IL-5, and IL-13 by T cells<sup>415, 416</sup>. CCL17, a chemokine that recruits Th2 cells into tissues, is increased in the serum and in biopsies of EGPA patients and is positively correlated with blood eosinophils and IgE<sup>413, 417</sup>. This increase in Th2 activity likely contributes to eosinophilia.

Blood eosinophils in EGPA show an activated phenotype expressing high levels of CD69 and CD11b<sup>414, 418</sup>. Moreover, they express IL-25, a cytokine that increases IL-4, -5, and -13 release from T cells. Serum IL-25 is increased in patients with active EGPA compared to inactive disease, or healthy controls. It is also detectable in eosinophils from lesional biopsies. T cells in these biopsies and in the blood express the IL-25 receptor IL-17RB<sup>419</sup>. This suggests a feed forward loop between eosinophils and Th2 cells in EGPA.

Neuropathy is a common symptom of EGPA<sup>32</sup>. Interestingly, different mechanisms lead to nerve damage depending on the presence or absence of MPO-ANCA. In patients with autoantibodies, MPO-ANCA-induced necrotizing vasculitis results in ischemic damage to the nerves<sup>420-423</sup>. In the absence of autoantibodies, massive eosinophil infiltration into the epineurium and occasionally endoneurium is observed. These eosinophils are degranulating and cytotoxic to nerves<sup>423, 424</sup>. Sometimes eosinophils form part of the inflammatory infiltrate surrounding necrotizing vessels<sup>423, 425, 426</sup>. This may accelerate damage of blood vessels because eosinophils were shown to be directly cytotoxic to endothelial cells *in vitro*<sup>84, 427</sup>. This damage may be mediated by ECP, which is deposited on endothelial surfaces in patients with eosinophilic endomyocarditis<sup>428-430</sup> or by MBP, which is cytotoxic *in vitro*<sup>84</sup>.

Eosinophil chemotaxis into affected tissues in EGPA patients occurs in response to eotaxin-3. Serum levels of eotaxin-3 are substantially higher in EGPA patients with active disease compared to those with inactive disease, healthy controls, or patients with other eosinophil-associated diseases<sup>212, 431</sup>. In contrast, there is no increase in serum eotaxin-1 or -2<sup>212</sup>. Eotaxin-3 is also readily detected in biopsies of affected tissues from EGPA patients<sup>212</sup>. Eotaxin-3 localizes to endothelial cells of small vessels, smooth muscle cells of small arterioles, the perineurium of the sural nerve, and the respiratory epithelium of the nose. An analysis of single nucleotide polymorphisms in the eotaxin-3 gene in 161 EGPA patients found no significant associations<sup>431</sup>, suggesting that eotaxin-3 polymorphisms may not be causal in EGPA.

The strongest evidence for a pathogenic role of eosinophils in EGPA comes from novel biological treatments that target IL-5 and thereby drastically reduce eosinophil levels. Two open-label trials with the anti-IL-5

antibody mepolizumab demonstrated its efficacy as a steroid-sparing agent and its ability to induce remission over 9 months<sup>432-434</sup>. Upon termination of mepolizumab treatment the majority of patients developed relapses. In one trial, eosinophil count and serum ECP were strongly correlated with disease activity<sup>434</sup>. A double-blind randomized placebo-controlled trial of mepolizumab in EGPA is currently ongoing (ClinicalTrials.gov identifier: NCT02020889).

Several key findings amount to moderate evidence for a pathogenic role of eosinophils in EGPA. 1) The number of eosinophils and serum ECP correlate with disease severity. 2) Eosinophil infiltration and degranulation in tissues causes organ damage. 3) A potential feed-forward loop between Th2 cells and eosinophils may propagate disease. 4) IL-5 targeted therapies showed beneficial effects.

### **Eosinophilic myocarditis**

Myocarditis is the inflammation of the heart muscle with or without damage or necrosis of adjacent myocytes in the absence of an ischemic event<sup>1</sup>. A wide range of causes from viral, bacterial and parasitic infections to toxic effects of drugs or hypersensitivity reactions can cause myocarditis and in many cases the etiology is unknown<sup>6</sup>. Autoimmune processes often play a role either causally or as post-infection autoimmunity: autoantibodies against cardiac antigens are present in the majority of myocarditis patients, myocarditis is associated with other autoimmune diseases, and some patients benefit from immunosuppressive treatment<sup>9, 12</sup>. Animal models provide further evidence for autoimmune mechanisms. Cardiac autoantibodies induce disease in rats, and immunization with cardiac myosin peptide in adjuvants induces experimental autoimmune myocarditis (EAM) in mice<sup>12</sup>.

Eosinophils form a major part of the inflammatory infiltrate in subtypes of myocarditis, namely in eosinophilic myocarditis and giant cell myocarditis. These subtypes are usually idiopathic. Eosinophilic myocarditis is associated with hypereosinophilic syndrome and EGPA but it can also develop in the absence of eosinophilia. About one third of EGPA patients and 20-50% of HES patients develop cardiovascular manifestations<sup>32-35, 164, 182</sup>. Myocarditis is more frequent in ANCA-negative EGPA patients<sup>32, 164</sup>. Parasitic

infections and hypersensitivity reactions to drugs are other potential causes of eosinophilic myocarditis<sup>14</sup>.  
<sup>24</sup>. Giant cell myocarditis and eosinophilic myocarditis are usually treated with strong immunosuppressive agents.

Eosinophils likely play a pathogenic role in the heart<sup>34, 40, 185</sup>. Eosinophil granule proteins are deposited in the myocardium during eosinophilic myocarditis and may be cytotoxic to cardiomyocytes<sup>39, 43, 44, 186</sup>. Eosinophils have also been proposed to activate cardiac mast cells<sup>435</sup>, or release pro-thrombotic tissue factor<sup>46</sup>. In HES, eosinophils are thought to damage the endocardium, which results in thrombosis and endocarditis and eventually leads to endomyocardial fibrosis and valvular complications<sup>33, 182</sup>.

Animal studies further strengthen the evidence that eosinophils contribute to pathology and mortality in eosinophilic myocarditis. Hypereosinophilic mice with transgenic expression of IL-5 (IL-5Tg) spontaneously develop eosinophilic myocarditis at a low frequency<sup>195</sup>. We found that induction of experimental autoimmune myocarditis (EAM) in these IL-5Tg mice reliably induces eosinophilic myocarditis with over 60% of the heart-infiltrating cells being eosinophils (see Chapter 4).

Induction of EAM in A/J mice causes myocarditis with numerous infiltrating eosinophils<sup>436</sup>. Blockade of IL-4 in this model reduces eosinophil infiltration and disease severity<sup>436</sup>. Induction of EAM in BALB/c mice that lack IFN $\gamma$  and IL-17A (IFN $\gamma$ <sup>-/-</sup>IL-17A<sup>-/-</sup>) results in severe eosinophilic myocarditis with about 50% fatality by day 21<sup>189</sup>. Ablation of eosinophils in these mice improved survival. In another model, natural killer cell depletion resulted in increased eosinophil infiltration in the heart and aggravated myocarditis. In eosinophil-deficient mice, however, natural killer cell depletion did not increase disease severity<sup>187</sup>. These results show that eosinophils are pathogenic in myocarditis.

A major burden of myocarditis lies in the sequela inflammatory dilated cardiomyopathy (DCM), which is the major cause of heart failure in patients under 40 years of age and has a poor 5-year survival rate of less than 50%<sup>138, 173</sup>. It is not known at what rate eosinophilic myocarditis patients progress to DCM or how this



rate compares to other myocarditis subtypes. Using the EAM model, we found that eosinophil-deficient mice are protected from DCM following myocarditis, while hypereosinophilic mice developed more severe DCM. This process was dependent on eosinophil-derived IL-4 (see Chapter 4). This suggests that eosinophils drive the chronic disease that ensues myocarditis.

Little is known about the mediators that induce eosinophil infiltration into the heart. We found increased expression of eotaxin-1 and eotaxin-3 in endomyocardial biopsies from patients with eosinophilic myocarditis compared to chronic lymphocytic myocarditis<sup>74</sup>. In the eosinophilic myocarditis mouse model of EAM in IFN $\gamma$ <sup>-/-</sup>IL-17A<sup>-/-</sup> mice, cardiac expression of eotaxin-1 and -2 is highly increased compared to naïve mice or WT controls<sup>74, 189</sup>. In this model, the eotaxin-CCR3 pathway is necessary for eosinophil trafficking to the heart during myocarditis<sup>74</sup>.

In summary, there is substantial evidence that eosinophils play a pathogenic role in myocarditis during the acute and chronic stage. Several studies in animal models offered mechanistic insight into how eosinophils contribute to myocarditis. It will be interesting to see if eosinophil-targeted therapies in patients with HES or EGPA will reduce the incidence of eosinophilic myocarditis in this high-risk group.

### **Neuromyelitis optica**

Neuromyelitis optica (NMO) is a demyelinating disease of the central nervous system that usually affects the optic nerve and spinal cord. Lesions are necrotic, cavitory, and infiltrated with macrophages and granulocytes. NMO is an autoimmune disease. Anti-aquaporin 4 (AQP4) autoantibodies are present in the majority of patients. These pathogenic antibodies are highly specific for NMO and are one of the features that distinguish it from multiple sclerosis. NMO patients often carry multiple other autoantibodies and there is a strong association with other autoimmune diseases. Moreover, NMO is much more common in women than men<sup>437</sup>.

NMO has only recently been distinguished from multiple sclerosis with eosinophil infiltration being one of the distinctive features<sup>438</sup>. The first description of eosinophil infiltration in NMO lesions was by Lucchinetti and colleagues in 2002. In analyzing lesions from NMO patient autopsies, they found eosinophil infiltration in early active lesions<sup>439</sup>. Eosinophils infiltration is located meningeal and perivascular in spinal cord lesions. Both intact and degranulating eosinophils are found<sup>439</sup>. Since this original observation, multiple studies have described eosinophil infiltration in the spinal cord, optic nerve<sup>440</sup>, brainstem<sup>441, 442</sup>, and cerebrospinal fluid<sup>443</sup>. Another study found that the cerebrospinal fluid from patients with NMO contains higher levels of eotaxin-2, eotaxin-3, and ECP compared to healthy controls or multiple sclerosis patients. In addition, stimulation of cerebrospinal fluid cells with myelin oligodendrocyte glycoprotein results in increased IL-5 production in NMO compared to controls<sup>444</sup>. Together, these studies clearly establish that eosinophils infiltrate and degranulate in NMO lesions, which suggests a pathogenic role for eosinophils.

A recent elegant study used in vitro experiments and a mouse model to determine the role of eosinophils in NMO<sup>445</sup>. Bone marrow-derived eosinophils exhibit antibody-dependent cellular cytotoxicity (ADCC) when co-cultured with a cell line expressing AQP4 in the presence of anti-AQP4. Similar effects of eosinophils are observed on spinal cord slice cultures. Stimulation of eosinophils with platelet activating factor, which induces release of EPX, results in damage to spinal cord slice cultures independent of anti-AQP4 antibody. The authors developed a mouse model of NMO by continuously infusing anti-AQP4 antibodies and human complement intracerebrally for 3 days. In this model, depletion of neutrophils, eosinophils or both reduces pathology. Likewise, eosinophil-deficient mice have less severe lesions. Induction of disease in hypereosinophilic mice results in more severe lesions with increased eosinophil and neutrophil infiltration<sup>445</sup>. This clearly established a pathogenic role for eosinophils in NMO and highlights mechanisms (ADCC and degranulation) by which eosinophils can damage neural tissues.

### Primary biliary cirrhosis

Primary biliary cirrhosis is a chronic disease of the small intrahepatic bile ducts that eventually leads to cirrhosis. It shows several hallmarks of an autoimmune disease: highly specific anti-mitochondrial autoantibodies, association with other autoimmune diseases, a female to male ratio of 10:1, and a strong genetic component<sup>446</sup>. Histologically, damaged biliary epithelial cells and infiltration of the portal area with plasma cells, T cells, NK cells, macrophages, neutrophils, and eosinophils are visible<sup>446, 447</sup>. Cytokine expression in the liver of primary biliary cirrhosis patients is similarly mixed. Compared to other liver diseases, increased hepatic expression of IL-5, IL-6, IFN $\gamma$ , TGF $\beta$ , and IL-2 has been noted<sup>448, 449</sup>. Recent studies also identified key Th1 and Th17 cytokines in the liver<sup>450</sup> and on blood cells<sup>451</sup>, and a decreased T regulatory to Th17 cell balance in peripheral blood cells<sup>452</sup>.

Patients with primary biliary cirrhosis have a higher frequency and increased absolute numbers of eosinophils in peripheral blood and the liver, particularly around damaged bile ducts<sup>453-456</sup>. Eosinophil infiltration is higher in the early stages of the disease (stages I-II versus III-IV)<sup>454, 456</sup>. Increased eosinophil infiltration was positively associated with liver IL-5 expression<sup>449</sup> and with mast cell infiltration<sup>455</sup>. Infiltrating eosinophils are degranulating, releasing ECP, MBP, and EDN, which can also be detected in the serum<sup>453, 456</sup>. Some patients have autoantibodies to eosinophil peroxidase (EPX)<sup>457</sup>, although it is unclear whether these have any pathologic relevance. Two of the established mouse models for primary biliary cirrhosis show eosinophil infiltration in the liver and could be useful for further studies on the role of eosinophils<sup>458-460</sup>.

Ursodeoxycholic acid (UDCA) is the only approved drug for primary biliary cirrhosis patients. UDCA can delay disease progression and improve liver biochemistry<sup>446</sup>. Of note, a higher frequency of blood eosinophils is associated with better response to UDCA treatment<sup>454</sup>. UDCA treatment decreases the frequency and number of eosinophils in the blood<sup>454, 461</sup> and in the liver<sup>456</sup> and decreases degranulation of tissue

eosinophils and serum MBP and EDN<sup>456</sup>. UDCA may suppress tissue eosinophilia by altering dendritic cells and the local cytokine milieu<sup>462</sup>.

In some cases, eosinophilia may precede the detection of liver pathology, suggesting that eosinophils are involved early in the disease processes. One study reports on 4 cases of asymptomatic women with eosinophilia detected during random investigation. All of them had elevated liver enzymes and were diagnosed with primary biliary cirrhosis<sup>463</sup>. In another patient eventually diagnosed with primary biliary cirrhosis, eosinophilia was detected 18 months prior to diagnosis but liver enzymes were still normal 12 months prior to diagnosis, suggesting that eosinophilia can precede overt liver pathology<sup>464</sup>. In conclusion, there is some evidence for a role of eosinophils in the early stages of primary biliary cirrhosis.

### **Other diseases**

Several rare diseases with a possible autoimmune etiology are associated with eosinophils. Usually only case reports or small case series are available for these diseases making it very difficult to rule out or assign a pathologic or protective role to eosinophils.

Eosinophilic cellulitis (Wells syndrome) is a very rare skin disease characterized by recurrent edematous erythema. Eosinophilic cellulitis is potentially associated with EGPA, HES, UC or other causes but the etiology is unknown<sup>465, 466</sup>. The typical histopathological sign is flame figures, the focal accumulation of disintegrating eosinophils and collagen fibers. Early stages are characterized by predominantly eosinophilic infiltration<sup>465</sup>. Blood eosinophilia is present in 15-67% of patients<sup>466</sup>. Blood eosinophils from patients with eosinophilic cellulitis express the high-affinity IL-2 receptor CD25<sup>467</sup>. In vitro, IL-2 treatment of CD25<sup>+</sup> eosinophils resulted in priming and increased release of ECP upon subsequent platelet-activating factor stimulation. This suggests that eosinophils in patients with eosinophilic cellulitis may degranulate more easily. Indeed, extracellular MBP staining is readily observed in flame figures<sup>468, 469</sup> and may be in amyloid form, a sign of large-scale degranulation<sup>470</sup>. Eosinophil chemotactic factors CCL17 and CCL24 have been detected in lesions<sup>471</sup>. It is possible that eosinophils play a pathogenic role through degranulation.

**Table 8: Autoimmune diseases with potential eosinophil involvement**

| <b>Disease</b>                     | <b>level of evidence</b> | <b>potential mechanism</b>  | <b>eosinophil recruitment</b>                                  | <b>tissue infiltration</b>                       | <b>blood eosinophilia</b>                           |
|------------------------------------|--------------------------|---|--|--|---|
| <b>Bullous pemphigoid</b>          | strong                   | eosinophil-derived proteases degrade extracellular matrix resulting in dermal-epidermal separation  | eotaxin-1, expressed by Keratino-cytes                         | yes  | yes, likely associated with disease severity        |
| <b>Inflammatory bowel diseases</b> | strong                   | release of EPX, MBP, IL-22BP; increase in mucosal barrier permeability; potential effects on enteric nerves                                       | eotaxin-1 (eotaxin-2 and -3), expressed by multiple cell types | yes, positively correlated with disease severity |   |
| <b>EGPA</b>                        | moderate                 | possible direct cytotoxic effects on endothelial cells, nerves, and other organs involved; pro-thrombotic effects                                 | eotaxin-3, expressed by various cell types                     | yes (diagnostic criterion)                       | yes (diagnostic criterion), increased Th2 cytokines |
| <b>Eosinophilic myocarditis</b>    | moderate                 | possible direct cytotoxic effects on myocytes or endocard; pro-thrombotic effects; mast cell activation; release of IL-4 promotes chronic disease | eotaxin-1, -3  | yes (diagnostic criterion)                       | not always present                                  |
| <b>Neuro-myelitis optica</b>       | strong                   | release of EPX killing astrocytes through antibody-dependent and complement-dependent cell mediated cytotoxicity                                  | eotaxin-2, -3  | yes, particularly in early lesions               |   |
| <b>primary biliary cirrhosis</b>   | weak                     | unknown, potential cytotoxic effects  |  | yes, particularly early stages                   | yes   |

Eosinophilic fasciitis is characterized by thickening and inflammation of the fascia resulting in painful swelling and progressive induration of the skin and soft tissues<sup>472, 473</sup>. The etiology of eosinophilic fasciitis is unknown. Autoimmune disease, infections, drugs, physical exertion and other factors are discussed as potential triggers<sup>474</sup>. There is no clear predominance by sex. Anti-nuclear antibodies are present in 15-20% of patients<sup>475</sup>. Blood eosinophilia is present in most cases (60-90%) and eosinophils infiltrate the fascia and sometimes the perimysium<sup>476-478</sup>. This increase in eosinophils is not always present and may be a transient feature. In one study, blood or tissue eosinophilia were not associated with the clinical outcome (cure versus residual fibrosis)<sup>477</sup>.

The fibro-inflammatory IgG4-related disease can affect multiple organs including the pancreas, salivary and lacrimal glands, lungs, retroperitoneum, and other tissues<sup>479, 480</sup>. While it seems to be an immune-mediated disease, no target antigen (autoimmune or microbial) has been identified<sup>481</sup>. The etiology and triggering factors are unknown<sup>480</sup>. High serum IgG4 is present in 60-70% of patients<sup>482</sup>, but it is unclear whether these IgG4 antibodies are directly pathogenic<sup>480, 481, 483, 484</sup>. Key histopathological features are a dense lymphoplasmacytic infiltrate, storiform fibrosis, and obliterative phlebitis<sup>479</sup>. Peripheral blood eosinophilia is found in about 30% of patients<sup>485</sup>. Eosinophils also infiltrate the tissues and are present in the majority of lesions. Eosinophil infiltration is usually mild to moderate but can be predominant in some cases<sup>479</sup>. To date, there is no clear evidence for or against a pathogenic role of eosinophils. However, several potential mechanisms have been hypothesized: antigen presentation, release of pro-fibrotic factors, and promotion of plasma cell survival for IgG4 production<sup>481</sup>.

### **Autoimmune diseases in patients with eosinophil-associated diseases**

Eosinophils are a key feature of asthma, hypereosinophilic syndromes and eosinophilic gastrointestinal diseases. An increased frequency of autoimmune diseases in patients with these eosinophil-associated diseases would be suggestive of a possible role for eosinophils in autoimmunity.

### **Hypereosinophilic syndrome**

There are numerous case reports of patients with hypereosinophilic syndrome who also suffer from an autoimmune disease including ulcerative colitis, autoimmune hepatitis, autoimmune thyroiditis, multiple sclerosis, systemic lupus erythematosus, antiphospholipid syndrome, myasthenia gravis, and rheumatoid arthritis<sup>486-501</sup>. Some of these patients had more than one autoimmune disease. From these case reports, the overall frequency of autoimmune diseases in HES cannot be determined. It is also not clear if HES precedes autoimmune disease or vice versa. In a trial of mepolizumab therapy for HES, 5 out of 78 patients under follow-up developed autoimmune diseases (rheumatoid arthritis, polymyalgia rheumatica, temporal arteritis, lichen planus, autoimmune thrombocytopenia)<sup>502</sup>. These diseases were likely revealed by the tapering of glucocorticoids but an effect of mepolizumab cannot be excluded. It seems that these are in excess of the expected prevalence of autoimmune diseases, however, future studies are required to determine if this is the case.

### **Eosinophilic gastrointestinal diseases**

Autoimmune diseases may be associated with eosinophilic gastrointestinal diseases. A recent literature review summarized case reports of autoimmune connective tissue diseases (SLE, rheumatoid arthritis, systemic sclerosis, and inflammatory myositis) in patients with eosinophilic gastroenteritis<sup>503</sup>. These patients were mostly female even though eosinophilic gastroenteritis shows a male predominance. The issue remains that case reports don't allow for any conclusion of association. A recent population based cohort study on eosinophilic esophagitis found that the risk of several autoimmune diseases was substantially increased in patients compared to controls<sup>504</sup>. Eosinophilic esophagitis patients had an increased risk of celiac disease, Crohn's disease, ulcerative colitis, rheumatoid arthritis, lupus, systemic sclerosis, Hashimoto's thyroiditis, and multiple sclerosis. No increased risk was found for pernicious anemia or vitiligo. Whether female compared to male eosinophilic esophagitis patients were more likely to also suffer from an autoimmune disease was not assessed. Genome-wide association studies have identified

risk loci for eosinophilic esophagitis that were previously associated with autoimmune diseases, suggesting a potential common genetic cause<sup>505</sup>. It will be interesting to see if eosinophilic esophagitis patients with autoimmune comorbidities differ from those without and whether an association of other eosinophilic gastrointestinal diseases with autoimmune diseases can be proven.

## **Asthma**

Eosinophils are a prominent feature of allergic asthma and, to a lesser extent, of non-allergic asthma<sup>506</sup>. A potential role for autoimmune processes in asthma has been proposed<sup>507</sup>. Patients with asthma carry autoantibodies more often than healthy controls<sup>508, 509</sup> and some studies have found a positive association of asthma with autoimmune diseases<sup>510-512</sup>. However, others report negative associations between these two conditions<sup>513, 514</sup>. It has also been suggested that sex hormones may contribute to asthma severity<sup>515</sup>. Non-allergic asthma has a female predominance, while allergic asthma does not<sup>516, 517</sup>. Whether autoimmunity plays a role in asthma pathology or is increased in affected individuals remains controversial. To date, there is limited evidence for increased autoimmune diseases in patients with HES, asthma or eosinophilic gastrointestinal disease and only one study that determined an increased risk in EoE patients. Future cross-sectional or cohort studies will be required to determine if the prevalence of autoimmune diseases is truly higher in patients with eosinophil-associated diseases.

## **Conclusions**

There is clear evidence for a pathogenic role of eosinophils in several autoimmune diseases. Protective functions of eosinophils have not been identified. Eosinophils contribute to autoimmune diseases in vastly different organs, from the central nervous system to the skin, gastrointestinal tract, and cardiovascular system. These include tissues where eosinophils reside in healthy individuals, such as the intestine, as well as those where eosinophils are usually absent, such as the heart or central nervous system. In all of these organs, eotaxins seem to be the main chemokines for eosinophil recruitment. Different eotaxins attract



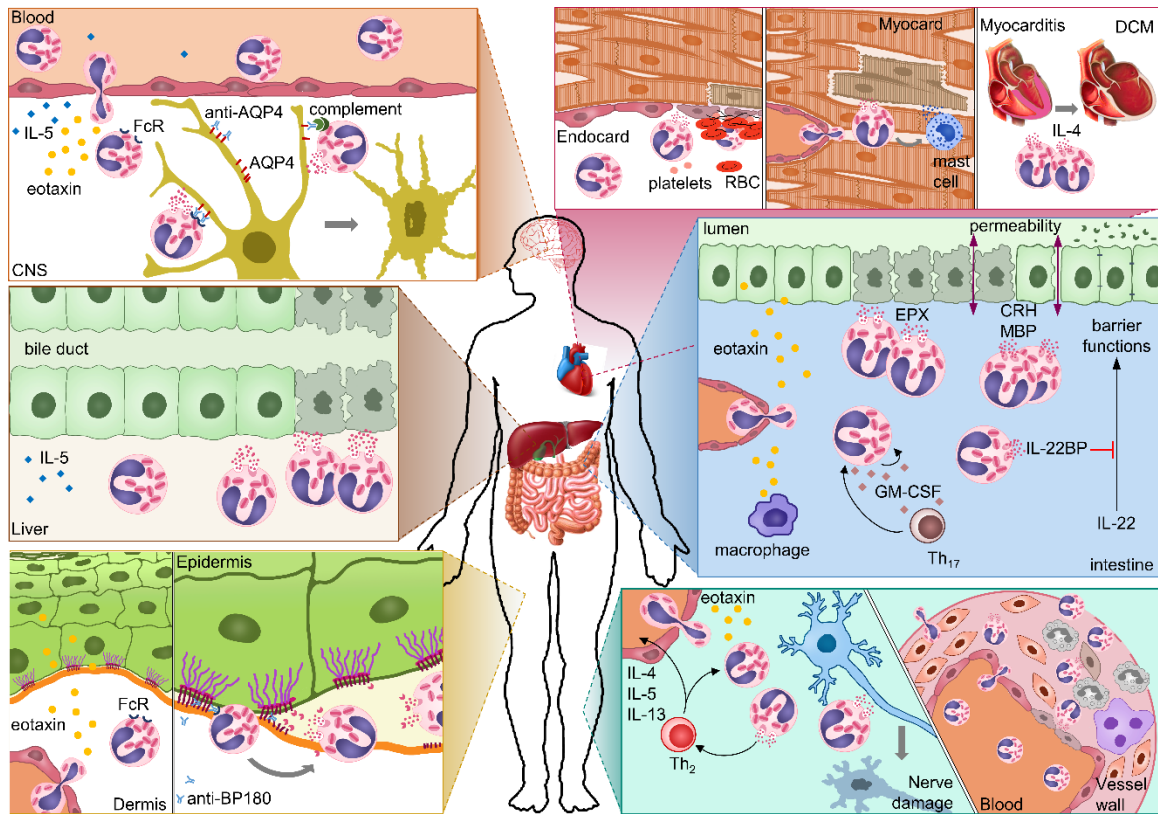
eosinophils to different tissues. Eotaxin-1 is essential for eosinophil trafficking to the intestine, while eotaxin-3 is most important in EGPA. Both eotaxin-1 and -3 attract eosinophils to the heart and eotaxin-2 and -3 recruit eosinophils to the central nervous system in NMO. Eosinophil infiltration into tissues is usually accompanied by eosinophilia, which may be transient, and is often caused by an increase in serum IL-5 or tissue IL-5. Other cytokines like GM-CSF may also be increased and contribute to eosinophilia. Compared to healthy controls, eosinophils from affected tissues or blood of patients show an activated phenotype, upregulating CD11b and CD69, and releasing cytokines such as IL-25, IL-6, IL-8, and IL-1 $\beta$ .

Multiple effector mechanisms have been identified (Figure 32). Degranulation of eosinophils is noted most frequently, perhaps because it is easily visualized by immunofluorescence or histology of biopsies. Degranulating eosinophils are often seen adjacent to dying cells, such as endothelial cells of the vasculature and endocard, nerve cells, dermis, and intestinal mucosa. As a result, direct cytotoxicity of eosinophils to other cells has been proposed as a mechanism in all autoimmune diseases discussed above. EPX, MBP and ECP all have strong cytotoxic properties and often multiple mediators are released. The ability of eosinophils to bind antibodies and subsequently degranulate and kill cells links the adaptive autoimmune response to eosinophil effector functions. Antibody-dependent cell mediated cytotoxicity by eosinophils was shown for NMO. Antibodies in BP likely cause degranulation of eosinophils and blister formation. Eosinophils are frequently associated with tissue remodeling. In BP, eosinophil-derived MMP9 and neutrophil elastase were shown to degrade extracellular matrix proteins resulting in dermal-epidermal separation. In ulcerative colitis, MBP and corticotrophin-releasing factor from eosinophils downregulate tight-junction proteins on epithelial cells, which decreases their barrier function. Eosinophil-derived cytokines modulating the function of other immune or stromal cells also play a role in autoimmune diseases. Eosinophil-derived IL-4 is important for chronic disease progression in myocarditis and IL-22BP blocks protective functions of IL-22 in UC. In several diseases, eosinophil infiltration was particularly pronounced in the early stages. This may hint to a role in initiation of the autoimmune response, a hypothesis that is difficult to prove in humans.

Particularly for rare diseases, the evidence for eosinophil involvement is mostly based on case reports, which makes it difficult to exclude associations by chance. Because autoimmune disease patients may receive many drugs, it is worth considering that hypersensitivity reactions to drugs are often accompanied by eosinophilia. On the other hand, it is difficult to ascertain the role of eosinophils in patients treated with glucocorticoids, which are highly effective at reducing eosinophil numbers in blood and organs<sup>518, 519</sup>. Eosinophils may be reduced to normal or below normal levels in patients under treatment, and this could mask any associations. Novel targeted therapeutics that affect only specific arms of the immune response and don't dampen eosinophils may reveal new associations.

### **Future research needs**

To verify some of the proposed mechanisms and potentially identify new mechanisms of eosinophil-mediated pathology or protection in autoimmune disease, animal models will aid greatly. The lack of in vitro or animal models has hampered research in several autoimmune diseases such as EGPA and primary biliary cirrhosis. In addition, epidemiological studies including larger patient cohorts will be required to determine whether autoimmune diseases are indeed increased in patients with eosinophil-associated diseases such as eosinophilic esophagitis, hypereosinophilic syndrome, or asthma. Lastly, viewing and analyzing autoimmune diseases with eosinophil-involvement as a group with possible shared mechanisms may advance our understanding and point to common processes.



**Figure 32: Possible mechanisms of eosinophil-mediated damage in autoimmune diseases**

In neuromyelitis optica, eosinophils damage astrocytes through antibody-dependent and complement-mediated cell mediated cytotoxicity. Eosinophil degranulation in damaged bile ducts was shown for primary biliary cirrhosis. In bullous pemphigoid, eosinophils release proteases that degrade the dermal-epidermal anchoring complex. Eosinophil infiltration in the heart results in damage to the endocard and myocard either directly or indirectly through mast cells. Eosinophil-derived IL-4 can drive progression from autoimmune myocarditis to dilated cardiomyopathy. In inflammatory bowel diseases, eosinophils can damage the mucosa through multiple mechanisms. Eosinophil granule proteins damage epithelial cells and increase epithelial barrier permeability. Eosinophil-derived IL-22BP blocks the protective effects of IL-22 on epithelial cells. GM-CSF may prolong survival and activation of eosinophils in the intestine. In EGPA, eosinophils damage nerves and blood vessels. Abbreviations: CNS, central nervous system; AQP4, aquaporin 4; FcR, Fc-receptor; BP-180, bullous pemphigoid 180; RBC, red blood cell; DCM, dilated cardiomyopathy; EPX, eosinophil peroxidase; CRH, corticotropin-releasing hormone; MBP, major basic protein; IL-22BP, IL-22 binding protein; GM-CSF, granulocyte-macrophage colony stimulating factor; Th<sub>17</sub>, T-helper 17 cell; Th<sub>2</sub>, T-helper 2 cell.

## Chapter 6: Conclusions and future directions

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We established a clear role for eotaxins and the eotaxin receptor CCR3 in eosinophil trafficking to the heart in a mouse model of eosinophilic myocarditis<sup>74</sup>. We identified the cellular source of eotaxin-1 as cardiac fibroblasts with interstitial localization in the heart and of eotaxin-2 as F4/80<sup>+</sup> macrophages localized at inflammatory foci. Expression of these chemokines is controlled by the Th2 cytokines IL-4 and IL-13. Furthermore, we demonstrated that eotaxins are also increased in patients with eosinophilic myocarditis and expression levels of eotaxin-1 and -3 are positively correlated with the number of heart infiltrating eosinophils. These results demonstrate that eosinophil trafficking to the heart is dependent on the eotaxin-CCR3 pathway in a mouse model and associated with eotaxin expression in patients with eosinophilic myocarditis. Our findings suggest that it might be possible to prevent eosinophil trafficking to the heart by blocking eotaxins. Monoclonal antibodies against eotaxin-1 are currently being evaluated in clinical trials (ClinicalTrials.gov identifiers: NCT01671956, NCT02226146).

Several questions remain open. We have not identified the cellular source of eotaxins in humans. It would be interesting to see whether the same cell types that express eotaxins in mice also produce eotaxins in humans. Moreover, our studies were done on a relatively small number of patients (16 with eosinophilic myocarditis and 14 with chronic lymphocytic myocarditis) and should be repeated with a larger cohort of patients and with additional groups of patients. For example, it would be of interest to see whether patients with giant cell myocarditis, who often have substantial cardiac eosinophil infiltration as well, also have increased expression of eotaxins.

Eotaxins are not the only chemokines that attract eosinophils. A limitation of our investigations is that we did not evaluate the expression of other chemokines like CCL5 or leukotrienes in eosinophilic myocarditis patients. The expression levels of these different chemokines should be analyzed and compared and their correlation to eosinophil infiltration should be assessed. These experiments would give insight into the importance of eotaxins and other chemokines for eosinophil trafficking to the heart. There are 2 eotaxins

in mice and 3 eotaxins in humans. We did not evaluate the relative importance of these chemokines. Others have shown that eotaxin-1 is important for eosinophil trafficking to the intestine<sup>69</sup> and eotaxin-2 for eosinophil trafficking to the lungs in allergic asthma models<sup>72</sup>. The relative importance of eotaxin-1 and -2 in experimental eosinophilic myocarditis could be tested by blocking each and both cytokines with monoclonal antibodies. More important, yet much more difficult to address, would be to understand which eotaxins regulate eosinophil trafficking to the heart in humans. Our studies showed tremendous increase in eotaxin-1 and -3 in eosinophilic myocarditis patients. It is possible that blocking only one of these chemokines will not be sufficient to prevent eosinophil trafficking. This might set trials of the anti-eotaxin-1 antibodies currently in development up for failure. Antibodies against other eotaxins are not in clinical trials at this time.

Another intriguing question is whether there is a difference between patients with and without peripheral blood eosinophilia. Our model of eosinophilic myocarditis in IFN $\gamma$ <sup>-/-</sup>IL-17A<sup>-/-</sup> mice has normal peripheral blood eosinophil numbers while that in IL-5Tg mice has hypereosinophilia. Eosinophil infiltration reaches over 60-80% in IL-5Tg mice but only 30% in IFN $\gamma$ <sup>-/-</sup>IL-17A<sup>-/-</sup> mice, yet eotaxins are dramatically increased (100-fold) in the hearts of IFN $\gamma$ <sup>-/-</sup>IL-17A<sup>-/-</sup> mice but only minimally (2-8-fold in the atria) in IL-5Tg mice. This leads to the hypothesis that high cardiac eotaxin expression is required for eosinophilic myocarditis in individuals with normal blood eosinophil numbers but not in those with hypereosinophilia. This hypothesis should be tested in patients as it may define the patient population for which eotaxin blockade may be useful.

Of course, the question remains whether the presence of eosinophils in the heart in patients with eosinophilic myocarditis actually contributes to pathology. While this is widely assumed it cannot easily be proven. New therapeutics such as anti-IL-5 and anti-IL-5 receptor antibodies are now being employed in eosinophil-associated conditions and offer a rare opportunity to assess the function of eosinophils in patients in vivo<sup>520</sup>. Anti-IL-5 antibodies substantially diminish eosinophil numbers by blocking

eosinophilopoiesis in the bone marrow. Anti-IL-5 receptor antibodies deplete eosinophils and other cell types expressing this surface receptor and can reduce eosinophils to undetectable levels in patients. These novel drugs have not yet been employed in patients with any form of eosinophilic myocarditis, but may reveal the function of this cell type once they are utilized.

In a different set of experiments, we sought to determine the role of eosinophils in myocarditis and its progression to DCM. Eosinophils were dispensable for myocarditis induction but required for progression to DCMi. Eosinophil-deficient  $\Delta$ dblGATA1 mice, in contrast to WT mice, showed no signs of heart failure by echocardiography. Induction of EAM in hypereosinophilic IL-5Tg mice resulted in eosinophilic myocarditis with severe atrial inflammation, which progressed to severe DCMi. This was not a direct effect of IL-5 as IL-5Tg $\Delta$ dblGATA1 mice were protected from DCMi while IL-5<sup>-/-</sup> mice exhibited DCMi comparable to WT mice. A caveat regarding the phenotype of the IL-5<sup>-/-</sup> mice is that the eosinophil numbers in the heart during EAM were not established. Nevertheless, we conclude from these results that eosinophils are dispensable for myocarditis initiation, drive progression of myocarditis to DCMi and cause severe myocarditis and DCMi when present in large numbers. These findings demonstrate clearly a pathogenic role for eosinophils in myocarditis.

There are some limitations of the IL-5Tg mouse model in that it does not recapitulate the exact pathology that is seen in patients with HES (outlined in the discussion of Chapter 4). Clearly there are large difference between mouse models and the pathophysiology in patients. An additional reason for the different histopathology may be the induction of disease in IL-5Tg mice. We used the EAM model which causes an autoimmune response against cardiac muscle. As expected, the IL-5Tg mice developed myocarditis. In humans, it is not clear what initiates the endomyocardial damage as some patients with HES develop these features while others live with hypereosinophilia but without cardiac manifestations for many years<sup>35</sup>. We are currently examining a cohort of IL-5Tg mice for the development of spontaneous cardiac disease. In these mice, we will determine pathological changes by echocardiography, electrocardiogram and histology

in comparison to age- and sex-matched WT littermates. It is possible that the spontaneous disease developing in IL-5Tg mice may replicate more features of cardiac pathology seen in HES patients. We have also done a microarray on cardiac tissues from IL-5Tg, WT and  $\Delta$ dblGATA1 mice and are currently analyzing the data. Even in naïve mice, there is a dramatic pro-inflammatory signature in IL-5Tg mice compared to the other two groups. Further analysis may reveal interesting processes resulting from the presence of large numbers of eosinophils in the heart.

We also identified the mechanism by which eosinophils promoted DCMi. Our experiments showed that eosinophils were the major IL-4 expressing cell type in the heart during EAM, IL-4<sup>-/-</sup> mice were protected from DCMi like  $\Delta$ dblGATA1 mice, and eosinophil-specific IL-4 deletion resulted in improved heart function. Thus, eosinophils drove progression to DCMi through their production of IL-4. These findings naturally lead to the question of what role IL-4 plays in human myocarditis. Little is known in this field and future studies should look into the expression of IL-4 in myocarditis and DCM patients to determine whether there is a correlation with disease progression. It would also be interesting to know whether patients with eosinophilic myocarditis have higher IL-4 expression compared to patients with lymphocytic or other types of myocarditis.

Our results did not rule out the possibility of additional eosinophil effector mechanisms in myocarditis and DCM. Eosinophil-specific IL-4 deletion resulted in a significant increase in ejection fraction compared to WT mice, but the difference was smaller than between WT and  $\Delta$ dblGATA1 or WT and IL-4<sup>-/-</sup> mice. There are two possible explanations for these results: incomplete deletion of IL-4 in eosinophils or additional mechanisms by which eosinophils promote DCM. We showed that the recombination in EoCre<sup>wt/tg</sup> mice is not complete by crossing these mice to the delete strain ROSA-DTA. Eosinophils were significantly reduced, but not completely absent. However, the possibility of other eosinophil-derived effectors that promote DCM cannot be ruled out.

Future experiments will focus on the function of IL-4 in DCM. The main questions that need to be addressed are: What are the target cells of IL-4? What effect does IL-4 have on these cells? How does that promote the development of DCM? We already know that IL-4 induces eotaxin expression in cardiac fibroblasts and macrophages. This suggests a possible feed-forward loop in which eosinophil-derived IL-4 promotes local eotaxin expression which in turn recruits more eosinophils into the heart. The effect of IL-4 on fibroblasts and macrophages is probably not limited to eotaxin production. Other target cells of IL-4 could be endothelial cells, which have been shown to respond with increasing vascular permeability and upregulation of cell adhesion molecules. Other immune cells in the lymphoid and myeloid lineages can also respond to IL-4. To address the question of target cells, IL-4R $\alpha$ 1 expression can be analyzed by flow cytometry as a first step. Once potential target cells are identified, the next step is to delete the receptor on specific cell types. For this, we have already acquired IL-4R $\alpha$ 1 floxed mice that can be crossed to mice expressing cell-specific Cre recombinase. The downstream effects of IL-4 and the link to the development of DCM remain entirely elusive at this point and may form the intriguing project of a future student.

By reviewing the role of eosinophils in other autoimmune diseases, we identified potential common effector mechanisms. We also found that eotaxins seem to play a key role in eosinophil localization to organs in all the disease where they were studied. Eosinophils may play an underappreciated role in certain autoimmune diseases and it may advance our understanding to compare and contrast these conditions. The development of new eosinophil-targeted drugs will offer exciting insights into the role of eosinophils in human disease and help answer some decades-old questions on the function of eosinophils in allergic responses, infections, and autoimmunity.



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- treated successfully with immune suppressive therapy and anticoagulation. *BMJ Case Rep* 2013 (2013).
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516. Knudsen, T.B., Thomsen, S.F., Nolte, H. & Backer, V. A Population-based Clinical Study of Allergic and Non-allergic Asthma. *Journal of Asthma* 46, 91-94 (2009).
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# Nicola Laura Diny

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Born on December 21, 1985 in Neunkirchen, Germany

### CURRENT POSITION

PhD Student, Department of Molecular Microbiology and Immunology, Bloomberg School of Public Health, Johns Hopkins University, Baltimore, USA 09/2011 - present

Thesis project: Eosinophils in autoimmune myocarditis and dilated cardiomyopathy

- Mechanisms of eosinophil-mediated damage and remodeling in chronic heart failure, using various transgenic mouse strains in experimental autoimmune myocarditis
- Translational studies on eosinophil chemotaxis in the heart in experimental model and patients

Advisors: Noel R. Rose, Daniela Čiháková

### EDUCATION

**Doctor of Philosophy** 05/2017  
Department of Molecular Microbiology and Immunology (expected)  
Bloomberg School of Public Health, Johns Hopkins University, Baltimore, USA

**Master of Science in Public Health** (concurrent with PhD) 05/2017  
Concentration: Global Disease Epidemiology and Control (expected)  
Department of International Health, Bloomberg School of Public Health

**Certificate, Vaccine Science and Policy** (concurrent with PhD) 08/2015  
Department of International Health, Bloomberg School of Public Health

**Master of Science in Molecular Medicine** 09/2011  
Charité University Medical School, Berlin, Germany

**Bachelor of Science in Biomedicine and Biotechnology** 09/2008  
University of Veterinary Medicine Vienna, Vienna, Austria

### PAST RESEARCH POSITIONS AND EXPERIENCE

**PhD Student**, Department of Molecular Microbiology and Immunology, Johns Hopkins University 09/2011 - present

Rotation projects:

- The EGO complex and Whi2 in TOR activation in response to Leucine (published in Mol Cell)

Advisor: Marie Hardwick

- Natural Cytotoxicity Receptors on Neuroblastoma

Advisor: Jelena Levitskaya

- Fibrosis in experimental autoimmune myocarditis in DC-depleted mice

Advisor: Noel Rose

**MSPH Student**, Department of International Health, Johns Hopkins University 08/2015  
Course project: Modeling adverse events in an experimental dengue vaccine trial using STATA statistical software - present

Advisor: Anna Durbin

|  |           |
|--|-----------|
| <b>Research Intern</b> , Pasteur Institute Shanghai, China   | 03/2011   |
| <u>Research Project</u> : Testing compounds with antiviral activity against Enterovirus 71   | - 06/2011 |
| Advisor: Ralf Altmeyer   |           |
| <b>Graduate Student Researcher</b> , Charité Medical School, Berlin, Germany   | 09/2008   |
| <u>Thesis Project</u> : The interferon stimulated gene 15 (ISG15) is essential for host defense against Coxsackievirus B3-myocarditis (published in Circulation)   | - 09/2010 |
| Advisor: Antje Voigt, Clinic for Cardiology and Angiology  |           |
| <u>Rotation projects</u> :   |           |
| <ul style="list-style-type: none"> <li>• PTHLH: a new candidate gene for Brachydactyly type E<br/>Advisor: Eva Klopocki, Institute for Medical Genetics</li> <li>• Functional characterization of mutant thyroid stimulating hormone receptors<br/>Advisor: Heike Biebermann, Institute of Experimental Pediatric Endocrinology</li> <li>• Stabilization of Mcl-1 by BH3-only proteins<br/>Advisor: Peter Daniel, Department of Hematology, Oncology and Tumor Immunology</li> </ul> |           |
| <b>Undergraduate Researcher</b> , German Cancer Research Center, Division of Developmental Immunology, Heidelberg, Germany   | 02/2008   |
| <u>Thesis Project</u> : In vitro culture system for medullary thymic epithelial cells  | - 08/2008 |
| Advisor: Bruno Kyewski   |           |
| <b>Undergraduate Researcher</b> , University of Veterinary Medicine Vienna, Vienna   | 10/2005   |
| <u>Rotation Projects</u> :   | - 01/2008 |
| <ul style="list-style-type: none"> <li>• Gene doping theme for "wahr/falsch inc." (Exhibition of science in Vienna)</li> <li>• Comparison of B-cells frequencies in different stages of maturation in swine<br/>Advisor: Armin Saalmüller, Department of Clinical Immunology</li> </ul>  |           |
| <b>OTHER EXPERIENCE</b>  |           |
| <b>Faculty Liaison</b> , Student Group, Department of Molecular Microbiology and Immunology, Johns Hopkins University, Baltimore   | 09/2014   |
|  | - 09/2015 |
| <b>Volunteer</b> with the Baltimore community organization Thread<br>www.thread.org  | 02/2014   |
|  | - present |
| <b>Social Coordinator</b> , Student Group, Department of Molecular Microbiology and Immunology, Johns Hopkins University, Baltimore  | 09/2013   |
|  | - 09/2014 |
| <b>Student assistant</b> to the program coordinator, Master's Program in Molecular Medicine, Charité Medical School, Berlin  | 10/2008   |
|  | - 04/2010 |
| <b>Volunteer</b> in an expedition of the humanitarian organization Surgical Eye Expeditions International to a hospital in Rundu, Namibia  | 07/2004   |
| <b>Technical assistant</b> , Ambulant Ophthalmic Surgery Centre, Saarlouis, Germany  | 01/2001   |
|  | - 09/2005 |
| <b>HONORS, AWARDS, AND FELLOWSHIPS</b>   |           |
| Excellence in Translational Research Award, Department of Pathology, Johns Hopkins University School of Medicine (poster on eosinophilic myocarditis in mice and patients)   | 03/2016   |
| First place, Laboratory Research category, Delta Omega Poster Competition, Johns Hopkins Bloomberg School of Public Health (poster on eosinophil migration to the heart)   | 02/2016   |
| American Heart Association Predoctoral Fellowship  | 07/2015   |
| (Proposal: Eosinophil-derived IL-4 drives inflammatory dilated cardiomyopathy)   | - 06/2017 |

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| Richard J. and Margaret Conn Himelfarb Student Support Fund (one award per year given to the best student in autoimmune disease research at Johns Hopkins Bloomberg School of Public Health) | 04/2015              |
| Excellence in Basic Research Award, Department of Pathology, Johns Hopkins University School of Medicine (poster on eosinophils in dilated cardiomyopathy)                                   | 03/2015              |
| Trainee Poster Award, American Association of Immunologists  | 03/2015              |
| Frederik B. Bang Award for Student Research, Johns Hopkins University Bloomberg School of Public Health (for a proposal on eosinophil-mediated cardiac remodeling)                           | 05/2014              |
| Student Conference Fund Award, Johns Hopkins Bloomberg School of Public Health Student Assembly  | 10/2014              |
| O'Leary-Wilson Fellowship, Johns Hopkins Autoimmune Disease Research Center  | 07/2014<br>- 06/2015 |
| Martin Frobisher Fellowship Fund, Johns Hopkins University Bloomberg School of Public Health   | 07/2013<br>- 06/2014 |
| Travel Grant Award, International Eosinophil Society (in recognition of oral presentation)   | 07/2013              |
| Katharine E. Welsh Fellowship in Immunology and Infectious Diseases, Johns Hopkins University  | 07/2012<br>- 06/2013 |
| PROMOS Stipend (Program for Increased Mobility of German Students), German Academic Exchange Service (for an internship at Institute Pasteur Shanghai, China)                                | 03/2011              |
| Bachelor Thesis Defense passed with distinction, University of Veterinary Medicine, Vienna, Austria  | 08/2008              |

#### PROFESSIONAL MEMBERSHIPS

|   |                |
|---|----------------|
| Trainee Member, American Association of Immunologists | 2015 - present |
| Student/Trainee Member, American Heart Association    | 2014 - present |
| Trainee Member, International Eosinophil Society      | 2013 - present |

#### PUBLICATIONS

**Diny NL**, Rose NR, Čiháková D. Eosinophils in autoimmune disease. Review. *Submitted*

Barin JG, Talor MV, **Diny NL**, Ong SF, Schaub JA, Gebremariam E, Bedja D, Chen G, Choi HS, Hou X, Wu L, Cardamone AC, Peterson DA, Rose NR, Čiháková D. Innate lymphoid cells couple the microbiome to autoimmune heart disease. *Submitted*

**Diny NL**, Baldeviano C, Talor MV, Barin J, Ong SF, Hou X, Schaub J, Bedja D, Kass D, Coppens I, Rose NR, Čiháková D. Eosinophil-derived IL-4 drives progression of myocarditis to inflammatory dilated cardiomyopathy. *J Exp Med*. In Press.

**Diny NL**, Hou X, Barin JG, Chen G, Talor MV, Schaub J, Russel S, Klingel K, Rose NR, Čiháková D. Macrophages and cardiac fibroblasts are the main producers of eotaxins and regulate eosinophil trafficking to the heart. *Eur J Immunol*. 2016 Dec;46(12):2749-2760 – *selected for back cover*

Barin JG, Talor MV, Schaub JA, **Diny NL**, Hou X, Hoyer M, Archer NK, Gebremariam ES, Davis MF, Miller LS, Rose NR, Čiháková D. Collaborative Interferon- $\gamma$  and Interleukin-17 Signaling Protects the Oral Mucosa from Staphylococcus aureus. *Am J Pathol*. 2016 Sep;186(9):2337-52.

Wu L, **Diny NL**, Ong SF, Barin JG, Hou X, Rose NR, Talor MV, Čiháková D. Pathogenic IL-23 signaling is required to initiate GM-CSF-driven autoimmune myocarditis in mice. *Eur J Immunol*. 2016 Mar;46(3):582-92. – *editorial choice featured "In this Issue"*

Ong S, Ligons DL, Barin JG, Wu L, Talor MV, **Diny NL**, Fontes JA, Gebremariam E, Kass DA, Rose NR, Čiháková D. Natural killer cells limit cardiac inflammation and fibrosis by halting eosinophil infiltration. Am J Pathol. 2015 Mar;185(3):847-61. – *editor's choice article*

Rahnefeld A, Klingel K, Schuermann A, **Diny NL**, Althof N, Lindner A, Bleienheuft P, Savvatis K, Respondek D, Opitz E, Ketscher L, Sauter M, Seifert U, Tschöpe C, Poller W, Knobloch KP, Voigt A. Ubiquitin-like protein ISG15 (interferon-stimulated gene of 15 kDa) in host defense against heart failure in a mouse model of virus-induced cardiomyopathy. Circulation. 2014 Oct 28;130(18):1589-600.

Teng X, Dayhoff-Brannigan M, Cheng WC, Gilbert CE, Sing CN, **Diny NL**, Wheelan SJ, Dunham MJ, Boeke JD, Pineda FJ, Hardwick JM. Genome-wide consequences of deleting any single gene. Mol Cell. 2013 Nov 21;52(4):485-94.

**Acknowledged contributions:**

Klopocki E, Hennig BP, Dathe K, Koll R, de Ravel T, Baten E, Blom E, Gillerot Y, Weigel JF, Krüger G, Hiort O, Seemann P, Mundlos S. Deletion and point mutations of PTHLH cause brachydactyly type E. Am J Hum Genet. 2010 Mar 12;86(3):434-9

**ORAL PRESENTATIONS (PRESENTER IS UNDERLINED)**

Diny NL, Hou X, Barin JG, Klingel K, Rose NR, Čiháková D. Eosinophil trafficking to the heart in eosinophilic myocarditis. Immunobiology Affinity Group Meeting, Brigham and Women's Hospital, Boston, June 2016.

Chen G, Wu L, **Diny NL**, Čiháková D. Signaling pathways in immune-mediated myocardial disease. Johns Hopkins University Cardiovascular Research Retreat, Baltimore, June 2016.

Diny NL, Barin JG, Baldeviano GC, Talor MV, Ong SF, Wu L, Rose NR, Čiháková D. Eosinophils and IL-4: Essential drivers of inflammatory dilated cardiomyopathy. 17th Annual Autoimmunity Day, Baltimore, June 2015.

Diny NL, Baldeviano GC, Barin JG, Talor MV, Legault J, Ong SF, Wu L, Rose NR, Čiháková D. Eosinophils are essential for progression of autoimmune myocarditis to dilated cardiomyopathy. 8th Biennial Symposium of the International Eosinophil Society, Inc., Oxford, July 2013.

Barin JG, **DinyNL**, Wu L, Cihakova D, Rose NR. Paradoxical interactions of IFN-gamma and IL-17 in autoimmune disease. 9th International Congress on Autoimmunity, March 2014.

**POSTER ABSTRACTS (PRESENTER IS UNDERLINED)**

Diny NL, Hou X, Barin JG, Talor MV, Schaub J, Klingel K, Rose NR, Čiháková D. Eosinophil trafficking to the heart in eosinophilic myocarditis requires the eotaxin-CCR3 pathway. Gordon Research Conference on Immunochemistry and Immunobiology, June 2016.

Diny NL, Hou X, Barin JG, Talor MV, Schaub J, Klingel K, Rose NR, Čiháková D. Eosinophil trafficking to the heart is dependent on the eotaxin-CCR3 pathway in eosinophilic myocarditis. Johns Hopkins Cardiovascular Research Retreat, June 2016

Diny NL, Hou X, Barin JG, Talor MV, Rose NR, Čiháková D. The eotaxin-CCR3 pathway is required for eosinophil trafficking to the heart in eosinophilic myocarditis. Immunology 2016, May 2016.

Diny NL, Hou X, Barin JG, Talor MV, Schaub J, Klingel K, Rose NR, Čiháková D. The eotaxin-CCR3 pathway recruits eosinophils to the heart during eosinophilic myocarditis. 18th Annual Department of Pathology Young Investigators' Day, March 2016.



**Diny NL**, Hou X, Barin JG, Klingel K, Rose NR, Čiháková D. The eotaxin-CCR3 pathway is required for eosinophil trafficking to the heart in eosinophilic myocarditis. Delta Omega Poster Competition, February 2016.

**Diny NL**, Barin JG, Talor MV, Rose NR, Čiháková D. Eosinophil trafficking to the heart in eosinophilic myocarditis is dependent on CCR3. 9th Biennial Symposium, International Eosinophil Society, Inc., July 2015.

**Diny NL**, Barin JG, Talor MV, Ong SF, Wu L, Rose NR, Čiháková D. Eosinophils and IL-4 are essential drivers of inflammatory dilated cardiomyopathy. Immunology 2015, May 2015.

**Diny NL**, Barin JG, Talor MV, Ong SF, Wu L, Rose NR, Čiháková D. Interleukin-4 in eosinophil-driven inflammatory dilated cardiomyopathy. Johns Hopkins Cardiovascular Research Retreat, May 2015.

**Choi HS**, **Diny NL**, Hou X, Barin JG, Talor MV, Schaub S, Čiháková D. Type 2 innate lymphoid cells in IL-33-induced eosinophilic pericarditis. 17th Annual Department of Pathology Young Investigators' Day, April 2015.

**Diny NL**, Barin JG, Talor MV, Ong SF, Wu L, Rose NR, Čiháková D. Eosinophils and IL-4: Essential drivers of inflammatory dilated cardiomyopathy. 17th Annual Department of Pathology Young Investigators' Day, April 2015.

Cottagiri M, **Diny NL**, Thomas D, Čiháková D, Rose NR, **Njoku D**. Hepatic IL-33, essential for immune-mediated drug-hapten hepatitis, is preceded by IL-4 and IL-6. Cell Symposia on The Multifaceted Roles of Type 2 Immunity, December 2014.

**Diny NL**, Baldeviano GC, Barin JG, Talor MV, Wu L, Ong SF, Legault J, Rose NR, Čiháková D. Eosinophils are essential for progression of autoimmune myocarditis to dilated cardiomyopathy. Gordon Research Conference and Gordon Research Seminar on Immunochemistry and Immunobiology, June 2014.

**Legault JA**, Ong S, Wu L, **Diny NL**, Nu QM, Barin JG, Talor MV, Gebremariam E, Čiháková D, Rose NR. The dual role of IL-6 in priming autoimmune myocarditis and driving inflammatory dilated cardiomyopathy. Johns Hopkins University Cardiovascular Research Retreat, May 2014.

**Diny NL**, Baldeviano GC, Barin JG, Talor MV, Wu L, Ong SF, Legault J, Rose NR, Čiháková D. Eosinophils are essential for progression of autoimmune myocarditis to dilated cardiomyopathy. Johns Hopkins University Cardiovascular Research Retreat, May 2014.

**Legault JA**, Ong S, Wu L, **Diny NL**, Nu QM, Barin JG, Talor MV, Gebremariam E, Čiháková D, Rose NR. The dual role of IL-6 in priming autoimmune myocarditis and driving inflammatory dilated cardiomyopathy. Johns Hopkins University Pathology Young Investigator's Day, April 2014.

**Diny NL**, Baldeviano GC, Barin JG, Talor MV, Legault J, Ong SF, Wu L, Rose NR, Čiháková D. Eosinophils promote acute myocardial inflammation and are essential for progression to dilated cardiomyopathy. 15th Annual Department of Pathology Young Investigator's Day, April 2013

**Diny NL\***, Opitz E\*, Klingel K, Riad A, Tschöpe C, Stangl K, Kuckelkorn U, Knobloch KP, Kloetzel PM, **Voigt A**. ISG-15 is Crucial in the Pathogenesis of Enterovirus Myocarditis. European Congress of Cardiology, August 2010. \*equal contribution

**Diny NL\***, Opitz E\*, Klingel K, Riad A, Tschöpe C, Stangl K, Kuckelkorn U, Knobloch KP, Kloetzel PM, **Voigt A**. ISG-15 is Crucial in the Pathogenesis of Enterovirus Myocarditis. International Symposium on Inflammatory Cardiomyopathy, Berlin 2010. \*equal contribution

**Gerner W**, Hammer S, Käser T, **Diny NL**, Pintaric M, Saalmüller A. Characterization of CD79 $\alpha$ +CD21- B cells in swine. Veterinary Immunology and Immunopathology 128 (2009) 211–347